HEPATITIS B ANTIGEN AND ANTIBODIES.

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PREFACE

It may be asked why the author of this thesis decided to venture into a study of 'Hepatitis B Antigen and its Antibodies' when it was obvious that this subject was, and would continue to be, the centre of the endeavours of established research workers from so many scientific disciplines all over the world, but there were several sets of circumstances that set the scene for this investigation.

In the first place there was an active team of research workers in Groote Schuur Hospital focussing their attention on liver disease and in particular on hepatitis. Their approach to the treatment of patients in acute hepatic failure had caught the attention of a wide international audience, and stimulated collateral research in other departments at the Medical School.

In the second place the author was introduced to the M.R.C. Virus Research Unit where he found an opportunity for analytical studies on new or incompletely characterized virus particles. There was a strong bias in this Unit for bio-physical, biochemical and serological measurements, with a host of techniques available which had proved to be successful in the study of a number of animal, plant and insect viruses. Another important factor was the presence of a superb electron microscopy facility.

Lastly, but important in the present context, the Virus Research Unit had a significant record of fundamental research in the field of vaccines and immune sera with particular reference to preparative techniques. Vaccines and antisera were just beginning to be talked about for hepatitis B virus infections when the present study was about to start, but long before the logistics of immunization programmes could be considered, a great deal had to be learned about the virus and its properties. Every bit of information however small, was going to be important. In this sense the author hopes that his contribution may be thought to have been worthwhile.

With the techniques available in the Virus Research Unit, some well known (density gradient zone electrophoresis, immune electron microscopy, affinity chromatography) and others developed in the Unit and modified for the present study (polymer precipitation with polyethylene glycol, ion exchange on formalin tanned gelatine granules and preparative immuno-absorbent electrophoresis) it was hoped to break new ground.

It was never the intention to duplicate in Cape Town what others had done elsewhere. Large scale surveys of the local populations were not to be part of the programme, but it was thought to be important that anything found to be of immediate applicability to clinical diagnostic problems should be exploited to the maximum.

Three examples of this became evident during the course of the investigations: i. the demonstrations of hepatitis B antigen in sera (and in a commercial sample of immune globulin being used in the Hospital) when standard serological tests including radioimmune assay had failed to detect it, ii. throwing light on the problem of demonstrating hepatitis B antigen in faecal samples and iii. the development of a technique for the demonstration of anti-HBc, the antibody to the core of the Dane particle. Restrictions had to be placed on the temptation to start using one or more of the multitude of new techniques reported in the literature over the period that this work was in progress.

While it would have been well beyond the scope of this thesis to review the enormous literature that has grown up about hepatitis B antigen and its antibodies, it was thought important to present a survey of the work of others, dealing with those aspects which have a bearing on the present study. With this in mind it was decided to consider the literature from four points of view. Historical perspectives. Changing concepts of the transmission of hepatitis B virus. Hepatitis B antigens and their intracellular sources. Hepatitis B antibodies, their specificities and their sequence of appearance.

The record of what was achieved in this investigation follows.

SUMMARY

The aim of this investigation was to study some of the biophysical, biochemical and serological properties of the antigen which had come to be firmly associated with hepatitis B infection even before the responsible virus had been identified with certainty. In anticipation of the Dane particle having this key role, it was given a high priority for detailed examination.

It was clear that the antibodies in patients recovering from acute hepatitis and in individuals who were chronic carriers of the hepatitis B antigen (HBAg) were directed against several antigenic components and these had to be distinguished from one another and separately assayed. A rather unique analytical situation therefore was presented by the serum of carriers in that the antigens and antibodies to be studied occurred together in the same starting material in mixtures of varying proportion, and sometimes as antigen-antibody complexes.

The techniques used, provided components of HBAg in a state of purity and concentration satisfactory for detailed examination. Polymer precipitation with polyethylene glycol 6 000 and ion exchange gave reproducible results in large scale fractionation procedures, and a specially designed apparatus (the "H-tube") provided purified samples of HBAg when only small amounts were

required.

A particular consideration in the purification and concentration procedures was to separate the specific immunoglobulins from components of the antigen without damaging either. This limited the variety of acceptable techniques but gel filtration and ion exchange proved successful to the extent that the discrete components of antigen could be used in the electron microscope for the detection of a specific antibody.

A careful study of the effects of various detergents and enzymes on the antigen had a number of important implications and applications. In the first place it was possible to disrupt the coats of the Dane particle to permit the release of the inner core components. This made it possible to show that the inner core was antigenically distinct and provided material for the detection of anti-HBc by electron microscopy.

The biophysical effects of the detergent on the antigen components was reflected in the altered charge density on the surface shown by the increased migration of the treated particles under standard conditions in a sucrose gradient subjected to electrophoresis. This surface alteration in the presence of proteolytic enzymes, circumstances somewhat resembling the conditions in the intestinal tract where bile salts and bacterial enzymes abound, resulted in the complete dissolution of surface

antigen leaving the resistant Dane core particles which could only be detected by immune electron microscopy. This result made a material contribution to the controversy which had resulted in a spate of papers with a confusing list of claims about the presence or absence of HBsAg in faecal samples.

Another was the demonstration of a new antigen and its antibody which were designated HB_{III} and anti-HB_{III} respectively. This was described as the third antigen antibody system since HBs anti-HBs and HBc anti-HBc were already well known. The antigen proved to be a cryptic one, not present on the unaltered surface of the HBAg but demonstrable with reproducible ease by detergent treatment. The demonstration that this reagent exposed $HB_{III}Ag$, led to greater clarity in the matter. There is a current paper associating $HB_{III}Ag$ with the e-antigen, and a recent letter to the Lancet (Neurath and Strick, 1977) suggests that e-antigen is a gamma globulin and that anti-e is an anti-gamma globulin. This is compatible with the concept of HB_{III} and anti-HB_{III}.

A contribution to the somewhat conflicting reports of the presence or absence of normal serum components on the surface of HBAg was made by a study which showed convincingly that an antigen, detectable by anti-human gamma globulin (and a number of other antisera directed to various loci of the gamma globulin molecule) using immune electron microscopy, was not found on the undamaged surface

of the antigen, but was regularly demonstrated after detergent treatment. Exposure of serum protein components (IgG, IgM and activated complement) was not confined to detergent effects but followed contact with caesium chloride, glycerol and other reagents used by investigators who had contributed some of the conflicting results.

The suggestion is made that the demonstration of these substances as an integral part of the HBsAg in the serum of chronic carriers of hepatitis B virus indicates a continual production of antibody to the surface antigen. This concept has recently begun to replace the older idea of defective production of anti-HBs by chronic carriers.

The investigation is thought to have been well worthwhile. Much of what is recorded in this thesis has been superceded by the tremendous pace at which new findings and techniques have continued to appear in the literature over the 4 years since the work was initiated. The participation in the several controversies at the time at which they were important has been a most rewarding experience.

Chapter 1

1.1. INTRODUCTION : HISTORICAL PERSPECTIVE

The emergence of the identity of the hepatitis B virus from the mists and myths surrounding the causes of infective jaundice, began with the recognition of a distinct infective agent in human blood.

Findlay and MacCallum (1937) associated the transmission of acute hepatitis with the administration of yellow fever vaccine, which had been stabilised with human serum. At about the same time hepatitis following the use of convalescent measles serum was reported (McNalty, 1937 and Propert, 1938).

Yellow fever vaccine administration to United States military personnel in the early years of the World War II was associated with 28 585 cases of jaundice of whom 62 died. An editorial in the Journal of the American Medical Association (1942) replied at length to allegations by the Chicago Tribune concerning the safety of the yellow fever vaccine and the validity of the safety trials of this product.

MacCallum (1972) related an incident that occurred in 1942 when Sir Winston Churchill nearly received yellow fever vaccine. MacCallum was asked whether Churchill should receive the vaccine for protection whilst in transit

through the Middle East and was able to advise against inoculation on the grounds of insufficient time for immunity to develop. Subsequently a very irate Director of Medical Services of the R.A.F. who had received vaccine from the batch that would have been used for Churchill, developed jaundice. Once the concept of serum hepatitis was accepted it was possible to relate the reported incidence of syringe-transmitted jaundice to the imperfect sterilization of parenteral equipment and the administration of human blood products rather than to the therapeutic agents employed (MacCallum, 1943 and 1945). Retrospective confirmation was available in a publication by Lurman (1885), whose lucid description of an outbreak of hepatitis amongst shipyard workers is reproduced in English by Zuckerman (1975), who correctly ascribed the outbreak to a batch of human lymph used for smallpox vaccination.

This impeccable logic was unmarred by ignorant postulates, a habit not infrequently encountered even in modern times, and the concluding sentence of his article notes that he was "not in a position to supply an explanation for this remarkable chain of cause and effect". This explanation was, at the end of World War II still only a well-informed postulate, but several workers, the first of whom was J.S.H. Gear in 1948, were able to demonstrate an immunological reaction between convalescent serum and either acute phase serum or liver extracts from hepatitis patients (Zuckerman, 1975) although it is

suggested that these were non-specific reactions (MacCallum, 1972).

It was not until 1963 that Blumberg et al., while working on the antigenic specifications of low density β-lipoproteins by assessing the antibody response of multiply transfused patients, detected a precipitin line by a micro-Ouchterlony technique between the serum of two of their panel of transfused patients and the serum of an Australian aboriginal. The antigen in this serum clearly differed from the serum β-lipoproteins (Blumberg et al., 1965) and was named the Australia antigen.

The distribution of this antigen amongst the normal population of various countries showed clearly that it occurred more commonly in tropical and crowded communities than in developed countries. However in America, where the incidence amongst the normal population was negligible, the antigen was found in a proportion of leukaemic patients and those with Down's syndrome and led Blumberg et al., (1965) to propose the test as an early diagnostic tool for the detection of leukaemia and to postulate that the antigen may be related to a viral agent responsible for the induction of leukaemia.

The pursuance of this side issue, no doubt associated with the institutionalisation of Down's syndrome patients and the need for repeated blood transfusion by leukaemics, was obviated by the observation and correct interpretation

from all over the world that occurred between 1963 and 1968, that Blumberg was honoured by the Nobel Prize Committee as co-recipient of the prize for Medicine in 1976.

Nonetheless it was Prince (1968) who firmly established the relationship between the Australia antigen and serum hepatitis and demonstrated that the antigen was not present in cases of infectious hepatitis. He investigated a group of hepatitis patients and was able to detect the antigen in the blood of 10/12 patients incubating serum hepatitis but not in the blood at any stage of the disease in patients who were judged to have developed infectious hepatitis. This was confirmed by retrospective examination of some of the Willowbrook sera for the presence of Australia antigen (Giles et al., 1969). The Willowbrook State School is an institution for mentally retarded children in which viral hepatitis had been endemic since 1949; with informed parental consent it was considered justifiable to gather data on the epidemiology and immunity of hepatitis by infectivity studies. These studies followed the work of MacCallum and Bauer (1944); MacCallum and Bradley (1944) on human volunteers in transmission experiments and of Havens et al., (1944); Stokes and Neef (1945) and Paul et al., (1945) and established two pools of icterogenic serum designated MS-1 and MS-2 as representative of the two strains of hepatitis endemic at Willowbrook. The pools corresponded to the classical infectious hepatitis (MS-1 strain of

of two fortuitous episodes. One of the Down's syndrome patients converted to an Australia antigen positive state while under investigation and was found to have developed hepatitis coincidental with the conversion. Then a laboratory worker for the group developed jaundice and again the antigen appeared in her blood concommitant with the clinical illness (Blumberg, 1968). An investigation of sera from cases of viral hepatitis showed that the antigen was detectable in 10.4% of cases, (Blumberg et al., 1967) and the association between the Australia antigen and hepatitis became more clear. However, the view of Blumberg's group in 1968 was that the antigen occurred in hepatitis, Down's syndrome, some forms of leukaemia and some apparently normal individuals resident in the tropics. They made the important observation that the antigen was persistent in some individuals and that it seemed that these people had an impairment of their immune mechanism which may be inherited. By rate zonal centrifugation of positive sera through sucrose density gradients, fractions were obtained that were reactive for the Australia antigen. These fractions when examined by electron microscopy showed the presence of 190 - 210 Angström spherical particles as well as filaments of the same diameter and varying in length from 500 - 2 300 Angström. These particles were found to be aggregated by prior incubation with human or rabbit antiserum to the Australia antigen (Bayer et al., 1968).

It was for this work, including both the discovery of the antigen and the vast amount of screening of sera

hepatitis A virus) and serum hepatitis (MS-2 strain of hepatitis B virus) and provided valuable information as to the infective dose, mode of infection and absence of cross immunity to the two strains as well as firmly characterising the clinical features of short (MS-1) and long incubation (MS-2) hepatitis (Ward et al., 1958; Krugman et al., 1959; Krugman et al., 1962 and Krugman et al., 1967). Studies at Willowbrook also adequately demonstrated the occurrence of what is still a major problem, the persistence of hepatitis B antigen. In the first series reported (Giles et al., 1969) 50% of the recipients of the MS-2 serum, whether they received the serum by intramuscular injection or whether they were given it orally, remained positive for Australia antigen for at least 3 years (the duration of the retrospective study.)

So far, reported progress seemed to indicate that hepatitis B virus was about to give up all its secrets as the association of a simple test and demonstrable 'virus' particle with clinical serum hepatitis enabled a large volume of mainly epidemiological data to be placed in correct perspective. Research efforts tended to centre on the virus itself and on the twin aspects of characterising the virus and propagating it in tissue culture. Initial attempts at assigning the small antigen to various classes of viruses were naive (Bayer et al., 1968) and further confused by the reported presence of about 5% RNA in isolated antigen (Joswiak et al., 1971).

Perhaps the most significant basic finding was that the Australia antigen appeared largely to be composed of serum protein; β-Lipoproteins-(LDL), 25 - 30%, IgG (the major protein component), B-1 a/c component of complement, albumin and transferrin were all identified by immunological means after three Tween 80 extractions of the purified antigen. (London et al., 1972). This observation received little attention at this time.

It is now known that the material subjected to such intensive analysis did not contain the infectious virus in any significant amount and it was the surface antigen alone that was analysed. The first description of a larger particle and the correct deduction that this represented the hepatitis B virus (HBV) was supplied by Dane et al., (1970). This large double shelled 47 nm particle has now officially been designated HBV (WHO: Weekly Epidem. Rec., No. 48, 1976) but will hopefully retain its common name "the Dane particle."

The evidence for allocating the role of virus to the Dane particle is in essence the result of the enormous amount of research both on the nature of the Dane particle and its morphology and occurrence as well as a study of the host immune response. This story really begins with the demonstration by Almeida et al., (1971) that the outer coat of the Dane particle, which had been shown to share a common antigen with the small particles (Dane et al., 1970), could be split from an internal component by detergent

treatment. These inner cores were complexed by post-hepatitis sera from patients with chronic renal failure (one had hepatitis 2 years previously) but not by the pre-hepatitis sera or by control sera. The outer coats of the Dane particle and the small antigen were not aggregated by the post-hepatitis sera. However, sera from a haemophiliac with antibody to Australia antigen produced complexes with both the internal component and the surface material of the Dane particle and with the small components. This led Almeida et al., (1971) to suggest that the antibody response to the surface antigen is short lived and that the "normal" antibody response in hepatitis virus infection is to the core component. It is interesting to note, although this is not commented on, that chronic renal failure is associated with a depression of the immune system and this may have been responsible for the "abnormal" response to surface antigen described in sera from their post-hepatitis cases. Of great significance was the realisation that the inner core of the Dane particle appeared to be identical in appearance with particles obtained from post mortem hepatitis livers prepared by Almeida et al., (1970).

1.2. CHANGING CONCEPTS OF THE TRANSMISSION OF HEPATITIS B VIRUS

Transmission of Hepatitis B

The original definition of hepatitis type B infection (MacCallum, 1947) inferred that the agent was transmitted

parentally by blood or blood products and this hypothesis presumably held for a large proportion of type B infections, until the need for adequate sterilization of instruments and routine screening of blood donors was realised.

The most recent situation is reflected by reports of hepatitis B in communities and by analysis of exposure factors (Heathcote and Sherlock, 1973) of index cases. In a series of 67 patients, 24% had likely parenteral exposure to hepatitis B and 40% had contact (non-parenteral) with HBAg carriers or acute hepatitis cases. Similar findings were reported for the West German district of Hannover by Mueller et al., (1975). The district included a city and its surrounds and involved a population of a little more than 1 million. Serum from all cases of suspected hepatitis were screened for HBAg and assessed for liver function abnormalities. HBsAg was found in 54% of confirmed cases of hepatitis with an age incidence maximal at 24 years and minimal before the age of 15 years. The HBsAg negative group on the other hand tended to be younger with the highest incidence of from 9 - 24 years, with a lowered incidence in the older age groups.

Evaluation of the HBsAg positive cases provided a history of blood transfusion in the preceding 6 months in only 13.5% with, at the other end of the spectrum, no history of surgery, dental treatment, injections, tatooing, drug addiction or contact (either occupational or social) with hepatitis cases in 21%. The remaining two thirds of

the HB Ag positive hepatitis patients had, within the six month period, been exposed to one or more of these hazards which may be considered unavoidable in developed communities. This epidemiology highlights some of the problems and gaps in the knowledge of the spread of infection and the immunity of a particular population group. Parenteral modes of spread are, in this study, possible in about 44%, and that presupposes that the listed exposures were indeed the source of the infection in every case.

Quite obviously transmission occurs in the majority of cases by means other than by inoculation with infected blood by exposure to the medical profession and its sharp instruments. Infected blood may be the mode of transmission in social contact, and a significant (10%) number had contact with carriers or acute hepatitis cases. The mechanism of spread may depend on either the accidental inoculation of traces, or the ingestion of a more substantial amount of infected blood (Krugman et al., 1967; Westwood et al., 1973). This has been shown possible by a number of reports and include early studies by MacCallum and Bauer (1944) in which MacCallum ascribes nasopharyngeal transmission of serum hepatitis to contamination of swabs with blood produced by the minute trauma of swabbing a throat (MacCallum, 1972).

Norwegian track finders hepatitis was abolished by the use of protective clothing which prevented scratches from undergrowth and inoculation by twigs and thorns or

pools of water freshly contaminated with the blood of preceding runners (Ulstrup et al., 1974). Towels and water in communal bathing houses after the race (Zuckerman, 1975) may have been a more likely method of inoculation with infected blood.

Barker et al., (1970) showed that 1.0 ml of a ten thousand-fold dilution of infective plasma given parenterally will transmit hepatitis, that a million-fold dilution may produce sub-clinical infection, and this indicates the possibility of transmission by blood or blood containing natural secretions by inocula as small as those mechanically transferred by mosquitoes (Prince et al., 1972; Papaevangelou and Kourea-Kremastinou, 1974; Purcell, 1975) or bedbugs (Mazzur and Jones, 1976). Even cockroaches from a dialysis unit were found to retain detectable HBAg for 10 days after ingestion of infected material, although this finding may be of academic interest only. The ritual mutilations so common amongst primitive people (tribal markings, circumcision) are well described (Blumberg and Hesser, 1975) and must result in transmission. The rituals of civilization (barbering, teeth brushing, ear piercing) where instruments are shared, may with less frequency be implicated in the spread of type B hepatitis.

Apart from cross inoculation by infected blood, there is a large volume of well documented information dealing with spread by contact alone (Lander et al., 1971; Ricci et al., 1973). The most readily acceptable category

relates to studies of families where a known carrier is involved (Szmuness et al., 1973; Irwin et al., 1974). Surveillance of renal dialysis patients who, because of the high risk associated with their presence in a dialysis unit, are relegated to home dialysis, has shown that household contact, but more particularly intimate contact, are clearly indicated as methods of spread. Sexual intercourse appears to be significant and studies of prostitutes reveal a higher incidence of anti-HBs when compared with the general population, an incidence which increases proportionally with length of service in that profession (Adam et al., 1974). Remarkably the carrier rate of HBsAg amongst prostitutes was not increased, suggesting an unusual form of successful vaccination. Both the antigen carrier rate, which was higher than the prostitutes', and seroconversions were higher among nuns than in a control group from neither profession. A point that seems to have escaped the attention of the authors is that by using the nuns as an index of non-venereal transmission, they had inadvertently chosen an Order which was engaged in active nursing in an institution in which there was a high incidence (40%) of endemic hepatitis.

There are reports of HBAg detected in semen, saliva (Tanno et al., 1972; Heathcote et al., 1974) and Mazzur (1973) has hypothetically suggested transmission by menstrual blood. All these factors support the findings of a higher incidence of transmission between sexual partners than between other family contacts (Hersh et al.,

1971; Mosley, 1972; Jeffries et al., 1973).

Non-sexual transmission of infection is well recorded, particularly amongst inmates of children's institutions (Krugman et al., 1967) and a striking example of this type of epidemiology was provided by a report (Vernon et al., 1976) on a study of hepatitis outbreaks among families who had adopted Vietnamese orphans. A high proportion (27%) of these orphans were found to be carriers (none were clinically ill with hepatitis) and the effect on the non-immune American community where the normal incidence of anti-HBs is 4.1%, was marked. Some 25% of the close contacts developed hepatitis B, this apart from an outbreak of hepatitis A associated with other numbers of these orphans.

If it is accepted that exposure to blood by other than the parenteral route, may transmit hepatitis B, and as an illustration the pattern of infection in renal dialysis units may be cited, where the incidence of hepatitis B per employee time is up to 150 times that of the incidence amongst the general nursing staff (WHO; Wkly. Ep. Record, 1973) then inapparent parenteral inoculation, ingestion of blood and possibly inhalation of blood aerosols may be considered to be important methods of transmission. Nevertheless the endemic situation in low socio-economic status of countries and institutions where evidence of exposure to the virus may be present in 25% (Mazzur and Jones, 1976) to 45% (Szmuness et al.,

1973) of the community may not be based on infection by blood, or even blood contaminated natural secretions alone.

A single report (Ogra, 1973) of a short appearance of HBAg in the nasopharynx of some patients may indicate a period of infectiousness. No mention of blood contaminating the nasopharyngeal samples was noted, but this may interestingly link with the MacCallum report (see MacCallum, 1972) of transmission of hepatitis by nasopharyngeal swabs. Nasopharyngeal secretion of infectious virus would be expected to occur during the prodromal period of infection and for this reason must be difficult to demonstrate, so that even a single report may be significant. There is no doubt that HBAg can be detected in saliva during the period of antigenaemia, and most reports (Ward et al., 1972; Brodersen et al., 1974) were assessed by relating the presence of HBAg with that of occult blood, and although blood was detected in many specimens, HBAg was detected in a significant number of persons whose saliva contained no detectable haemoglobin. Probable transmission is recorded following a bite from a retarded child whose saliva was positive for HBsAg and negative for occult blood (CDC: MMWR, 1974).

Whether this is a significant epidemiological finding is doubted by Marcolongo et al., (1973) who were only able, by counter-electrophoresis, to detect HBAg in saliva in one out of twentyfive patients with acute type B

hepatitis. Interestingly, sweat was found to be positive in 40% of cases by the relatively insensitive technique of immunodiffusion (Telatar et al., 1974) but it must be remembered that by the nature of the tests used for detecting antigen in these secretions, a positive result is only dependent on the presence of surface antigen. This does not necessarily indicate the infectivity of positive samples, merely the presence of antigenic surface determinants. Indeed it would be expected that the HBV itself may only enter secretions as a result of leakage. Most reports stress the low level of HBAg in concentrated specimens as compared with the level detected in the serum of these patients.

A single report of hepatitis B following accidental contamination of the eye of a nursing sister indicates that infective blood may transmit the disease through the conjunctiva (Kew, 1973) but tears have been found to contain HBAg (Vittal et al., 1974).

The infectivity of urine and faeces must be considered separately as the incidence of hepatitis B infection is increased in lower socio-economic populations (Cherubin et al., 1972), a finding usually related to faecal-oral spread, and reports from Willowbrook (Krugman et al., 1967; Giles et al., 1969) amply indicate the high endemic rate prevalent in the institution due to natural spread.

There is no direct confirmation of spread by this

route, early transmission studies are confused by the lack of specific tests for separating A and B type infections, and although the presence of serologically detectable antigen has been reported (See Chapter 9) and morphological antigen isolated from faeces (Tripatzis, 1972; Sonnabend et al., 1972) and detected in urine (Heathcote et al.,1973) there is no recent study in the available literature of transmission experiments. MacCallum and Bradley (1944) reported transmission of hepatitis by ether-treated vanilla flavoured faecal homogenates in 3/26 cases. Unfortunately the source of the material is only listed as "from infective hepatitis cases", and the incubation periods are between 27 - 31 days, indicating transmission of hepatitis A. Indeed there is some evidence that among healthy adults there is little person-to-person spread, in particular troops receiving the hepatitis B contaminated yellow fever vaccine and an outbreak of hepatitis following plasmaphoresis experiments in 5 prison populations produced no significant lateral spread (Mosely, 1972).

Shellfish are known to concentrate toxins from seawater and outbreaks of hepatitis A have been associated with clams and oysters harvested from contaminated water (Dougherty and Altman, 1962, and others). Blumberg and associates (Mahoney et al., 1974) tested clams from bacterially polluted areas for hepatitis B antigen which was found in one area only, an area polluted with untreated effluent from a small hospital. It is tempting to assume that hepatitis B antigen is not present, in sewage even

when concentrated by bi-valves, except where contaminated with hospital efflux.

After developing a sensitive assay for detecting HBAg in dilute fluids (Grabow and Prozesky, 1973) Grabow et al., (1975) were not able to detect HBAg in 2 litre volumes of raw sewage nor in faeces (80-150 g) or urine (1 litre) from high titre HBAg positive patients. Blumberg and his associates (Mazzur et al., 1973) had shown a loss of immunological reactivity when HBAg was incubated with pseudomonas cultures and this was confirmed and associated with, in addition, a sensitivity to carboxy peptidase A demonstrated both for surface antigen and Dane core. Certainly the antigen is immunologically altered, whether it remains infectious (Mazzur et al., 1973) would, in the light of present knowledge of the function of the Dane core, seem to be highly unlikely. It would appear that under these 'in vitro' conditions HBV and HBsAg are destroyed and that any transmission of the disease by the faecal-oral route, may be related to contamination of the excreta by blood or serum. As with saliva, transmission studies in chimpanzees would provide the only method of assessing infectivity.

1.3. HEPATITIS B ANTIGENS AND THEIR INTRACELLULAR SOURCE

In spite of this early exposition of the separate

specificity of the core of the Dane particle (HBc), immunofluorescent studies of hepatocytes from livers of patients with Australia antigen positive hepatitis were clouded by the use of antisera which contained either anti-HBs or both anti-HBs and anti-HBc (Hadziyannis et al., 1972; Nielsen and Elling, 1971). Brzosko et al., (1973) however were able to demonstrate the duality of the antigen by using serum from patients with acute viral hepatitis and chronic aggressive hepatitis to show nuclear fluorescence, and sera from hepatitis B antibody positive persons and hyperimmunised animals to show cytoplasmic fluorescence. This information is consistent with the knowledge that Brzosko and his associates (1973) had selected and prepared antisera with anti-HBs and anti-HBc specificity and that these antisera firmly located core antigen in the nuclei and surface antigen in the cytoplasm. The location of core antigen in the nuclei of infected hepatocytes had been indicated by Nowoslawski et al., (1970) and Nelson et al., (1970) who described virus-like particles in the nuclei of hepatocytes by electron microscopy in ultra—thin sections, and this was confirmed by Blumberg's group (Huang, 1971) and serologically identified with ferritin and fluorescin labelled guinea pig anti-HB serum (Huang et al., 1972).

Surface antigen in the cytoplasm of hepatocytes has been regularly demonstrated by immunofluorescence and the pattern of fluorescence, whether it be focal, diffuse or solitary correlated with clinical and histopathological

patterns of acute hepatic necrosis, chronic active hepatitis and chronic persistent hepatitis (Roos et al., 1976). In addition, a correlation has been shown between eosinophilic "ground glass" hepatocytes in conventionally stained sections by light microscopy (increased smooth endoplasmic reticulum), orcein or aldehyde fuchsin positivity (Shikata et al., 1974; Deodhar et al., 1975) and anti-HBs fluorescence. Confirmation that this was the site of surface antigen production using electron microscopic examination of ultra-thin sections was initially less convincing, especially in view of the large amount of surface antigen present in the peripheral blood.

The early demonstration of occasional coated particles, similar in size and morphology to the Dane particle, which were found in phagosomes and cell sap were not convincing and may represent artifactual positioning during sectioning (Huang, 1971) but Stein et al., 1972(a) and (b) showed convincingly that the dilated cisternae of the endoplasmic reticulum of hepatocytes from 2 healthy carriers contained filaments which on cross-section appeared to have an electron dense centre. This "owl eye" appearance was similar to that of positively stained sectioned antigen obtained from serum.

Further work by Huang et al.,(1974) led to the distinction of the "owl eye" appearance of filaments in cross section from the appearance of Dane particles in cross section, both of which were reported in the endo-

plasmic reticulum. Homogenates of the same liver preparations were negatively stained and intertwined bundles of filaments, tadpoles and Dane particles were isolated and found to be identical both morphologically and serologically with those occurring in serum.

Gerber et al., (1972, 1973, 1974) reported similar findings and in addition found that the percentage of hepatocytes affected varied from 5% to virtually all the hepatocytes observed. Up to ten circular bodies were observed within the same cisterna and were usually accompanied by a flocculant material. Peroxidase labelled anti-HB reacted with the membrane of the smooth endoplasmic reticulum and with the intra-membranous particles. The authors suggest that the use of immunologically specific labelled antisera for core and coat would confirm their findings, but if their findings are judged in conjunction with those obtained by other techniques it must be clear that a site of surface antigen production has been satisfactorily demonstrated.

The enormous mass of circulating surface antigen, when contrasted with the paucity of morphological antigen in hepatocyte cytoplasm in early electron micrographs, raised some doubt as to whether the liver alone produced all the antigen. The half life of surface antigen is unknown, but this apparent inconsistency becomes more plausible when it is considered that there is both considerable proliferation of the endoplasmic reticulum in infected cells (Popper and Schaffner, 1976) and that the surface

area of endoplasmic reticular membrane has been calculated to be approximately 8 - 13 m^2 for each 1.0 ml of normal liver tissue (Weibel et al., 1969).

A recent publication confirms these findings (Trump et al., 1976) and again demonstrates, by sections of infected hepatocytes, the proliferated endoplasmic reticulum, the profiles and cisternae of which contain circular and lamellar profiles 20 - 25 nm in diameter.

The reference by authors to circles, circular or ellipsoid profiles or bodies points to the appearance of filamentous antigen in cross section, which on positive staining seems in some instances to have a central electron dense dot (Gerber, 1974). It is this appearance which in earlier works led to the discussion of the relationship of the coated particles to Dane particles.

It seems obvious now that the cisternae contain packed filaments of HBAg positive material, arranged in twisted cords, and that some of these filaments are in tadpole form with terminal widening of the coat to enclose a core. Coated particles are also found and on negative staining of material produced from these cells by disruption (Huang et al., 1974) it would appear that these structures correspond to the Dane particles, tadpoles and filaments described in serum. Small spheres, so numerous in sera, were not obvious in homogenates of liver cells and may only be formed after release from hepatocytes.

The mechanism of production of this material is not known, and the original hypothesis of Dane et al., (1970) - that this represents excess coat production has not been invalidated by recent work.

In considering the chronological events leading to an unravelling of the mysteries of the source of this unusual virus, progress towards the development of a tissue culture system and animal model for propagation and isolation of the virus must be mentioned. The benefits of such a system would be immense, both as a tool for further investigation of viral morphogenesis and for the evolution of vaccines. Many attempts have been made, both pre- and post-Australia antigen era, to isolate an infectious agent from cases of hepatitis in a variety of tissues from chick embryo to tobacco plant. (MacCallum and Bauer 1944; Banatvala, 1973). Growth of hepatitis B virus in culture has been reviewed (Nature, 1974; Zuckerman, 1975) but in spite of reports of individual successes in liver cell cultures and human embryo organ culture as judged by immunofluorescence or serological tests (Smith and Francis 1972; Noyes, 1973; Shikata, 1973) and additionally by electron microscopy (Zuckerman et al., 1972; Zuckerman and Bird 1973; Panouse-Perrin et al., 1973), the results have not been generally reproducible. Recent comment that "the successful cultivation of hepatitis B virus in tissue or organ culture has not yet been achieved" (Krugman, 1975), may be unfair to some individuals, does nevertheless reflect the generally held view.

The report of an established cell line from a primary liver cell carcinoma (Alexander et al., 1976) from a patient whose serum was positive for HBsAg and the identification by radio immunoassay and reversed cell haemagglutination techniques of hepatitis B antigen in the uninoculated cell culture supernatant fluid (McNab et al., 1976) may provide a means for investigating the host/virus relationship.

Non-human primates provide an alternate to human volunteers for transmission and immunity studies of the hepatitis B virus and fortunately, after initial confusion occasioned by the later demonstration of 6 - 12% pre-existing immunity of captive chimpanzees,(WHO 1973), this animal shows mild symptoms of infection following inoculation with the virus.

Maynard et al., (1972) were responsible for the initial demonstration of infection with hepatitis associated antigen by sero-conversion and this antigen was demonstrated for them in the serum of an inoculated chimpanzee by electron microscopy. Barker et al., (1973) showed the presence of HB antigen specific fluorescence in the hepatocytes of an infected chimpanzee and subsequently continued transmission experiments establishing the parameters of the mild illness produced. At this stage the significance of the duality of HBAg was more fully realised and in 1974 the Barker group reported progress as a result of chimpanzee experiments in the study of the

second type of antibody (anti-HBc) and its association with viral replication. Small amounts of core antigen had previously been available only from liver biopsy material or from preparations from serum and this had hampered the development of serological tests for the presence of anti-HBc. A cyclophosphamide suppressed chimpanzee died from pneumonia after being experimentally infected with hepatitis B and the liver of this animal contained numerous intra-nuclear cores, which when extracted, provided sufficient core antigen for radio-immunoassay and complement fixation. This allowed the retrospective study of sera collected weekly from human infectivity studies conducted in 1951 - 1954. The patterns of serological response were established for acute infection, abortive primary infection, development of the carrier state and anamnestic response (Hoofnagle et al., 1973; Hoofnagle et al., 1974; Hoofnagle et al., 1975).

A further link in the chain of evidence that the HBV is present in Australia antigen was provided by the detection of endogenous DNA polymerase in crude pellets of antigen (Hirschman et al., 1971) and this activity was shown to be associated with cores obtained from detergent treated Dane rich sera by Kaplan et al., (1973) who demonstrated a sub-population of Dane cores in the sucrose density gradient fraction containing the polymerase activity. Inhibition of enzyme activity by actinomycin D and daunomycin indicated that the polymerase was DNA

dependent, although Hirschman and colleagues had suggested that the reaction was RNase labile, and confirmation of this came from the detection of actinomycin D, poly(dA-dT)·poly(dA-dT) inhibited DNA polymerase activity in the serum of experimentally infected chimpanzees (Bradley et al., 1974). These workers were able to correlate the appearance of polymerase activity in the serum with infection, and demonstrated its appearance in several peaks prior to evidence of liver dysfunction and the appearance of circulating HBsAg.

Some of the co-workers in the original detection of DNA polymerase, (Robinson et al., 1974) were able to react DNA polymerase with purified cores and obtain core structures with a radioactive DNA product. After extraction with sodium dodecyl sulphate examination of the banded radioactive material from a caesium chloride gradient by flotation technique produced the first electron micrographs of the small double stranded circular DNA molecule. Circular DNA was also released from cores without reaction with synthetic label. After the necessary steps of first treating the core preparation with DNAse to remove unprotected DNA, disruption of the core provided circular DNA molecules. The open configuration of the DNA molecule, and thermal denaturation and enzyme studies additionally indicated both the double strand and a G+C content of 49%. The length of the molecule was determined by measuring 225 circular strands to give a mean of 0.78μ m (780nm) to correspond to a molecular weight of about 1.6×10^6

daltons. This is smaller than the double stranded DNA of any known virus and the genetic information carried must be very limited, It is suggested that this may only be sufficient to code for the proteins associated with coat antigenicity, the nucleocapsid antigen and the DNA polymerase. The additional peptides identified in coat material would seem to be beyond the capacity of the genome. Hung et al., (1975) have essentially confirmed these findings by reporting that the tritiated circular DNA from Dane particles hybridizes with free DNA in HBs carrier plasma but not with DNA prepared from normal liver extracts.

All the evidence for considering the core of the Dane particle to be the nucleocapsid of the HBV is present and indeed the title has been officially conferred (WHO: 1976). It is obvious however that this infectious agent, the subject of an immense volume of research compressed into less than a decade, will continue to absorb the interest of investigators for a long while to come. At the present time, because of some of the anomalies described, there is talk of "helper viruses", hypothetical "infectious particles with larger DNA molecules","excess coat protein production" and much reference to this "unique virus", the signs of indecision and confusion that tend to accompany efforts to explain phenomena as yet only partially understood.

1.4. HEPATITIS B ANTIBODIES, THEIR SPECIFICITIES AND THEIR SEQUENCE OF APPEARANCE

The pattern of serological response to infection by the HBV has been well documented by (Hoofnagle et al., 1973, 1974, 1975) and by Krugman, Ward and Giles from Westbrook (reviewed by Krugman, 1975). Antibody to the surface antigen, anti-HBs, usually becomes detectable in serum after the disappearance of circulating HBsAg and coincident with the abatement of jaundice or biochemical dysfunctions. The development of more sensitive assays showed that although the titre may be low, most patients with acute hepatitis develop an antibody response to HBsAg even although this may be months after the acute disease (Robinson and Lutwick, 1976). It has been postulated recently (Melnick et al., 1976) that anti-HBs may be produced at low levels continuously throughout the stages of hepatitis, but the formation of immune complexes inhibits the manifestation of antibody until the level of HBsAg falls. Evidence to support this concept is presented elsewhere in this thesis.

The complement fixation test developed by Hoofnagle et al., (1973) for measuring anti-HBc levels had a profound influence on the understanding of the temporal relationship between persistent infection in the carrier and the antibody response. It was clearly shown that anti-HBc was associated with continuing viral replication and that this antibody developed early in infection. The detection of

anti-HBc seems to coincide with liver damage, but is preceded by the appearance of HBsAg in the circulation. All chronic carriers (persistence of HBsAg in the serum for more than 3 months) are shown to have high levels of anti-HBc, although not all have easily detectable evidence of liver damage. Mild infections characterized by anicteric hepatitis and a shortened period of HBs antigenaemia produce a transient level of anti-HBc as evidence of HBV replication. Certainly no anti-HBc response has been noted in patients who have received inactivated virus (Krugman et al., 1974). In their description of the serological responses to HBV infections determined retrospectively by titration of anti-HBs and anti-HBc, Hoofnagle and his co-workers (1975) included the pattern of response resulting from re-exposure to the virus three months after an attack of acute hepatitis. This demonstrated an anamnestic response to coat material alone, but does not necessarily show that this is the protective antibody, certainly no evidence of core replication, as judged by a rise in anti-HBc titre, was found in these cases.

Recent third generation tests for anti-HBc (Robinson and Greenman, 1974; Purcell et al., 1974) have largely confirmed the findings of Hoofnagle and Krugman, but an immune adherence test for anti-HBc developed by Mayumi and associates along the lines of an original third generation test for surface antigen (Mayumi et al., 1971) has shown in contrast to the American reports, that anti-HBc is more prevalent than anti-HBs amongst healthy Japanese blood

donors. This may be explained by the increased sensitivity of the test (approximately 10-fold) or by differences in the carrier rate of the two countries (Tsuda et al., 1975). As a routine test it may detect the HBsAg negative/anti-HBs negative convalescent donors mentioned previously who may still have low levels of circulating immune complexes and be responsible for some of the 59% (Prince et al., 1975) or 50% (Alter et al., 1972) of HBAg negative blood units that transmit hepatitis B infection.

It is becoming increasingly clear that the immunological competence of the host determines the pattern of the response to infection by the hepatitis B virus. The host with normal immunological mechanisms will clear the virus completely after an acute clinical illness, but the host with a less efficient immune mechanism may present with a less acute hepatitis, with slight or even no jaundice and is more likely to become a chronic carrier of the hepatitis B antigen (Dudley et al., 1972a and b). The mechanism of this immunological reactivity has not been demonstrated, but immunofluorescent studies have shown that granular deposits of membrane-fixed IgG are present on isolated hepatocytes prepared from the livers of patients with HBAg positive acute hepatitis (Hopf et al., 1976). Effector lymphocytes (K cells) may be responsible for this antibody dependent cytotoxicity (Sherlock, 1976). Humoral antibody may be considered an important and readily detectable marker of the immune response and the evaluation of the specificities of the

immunoglobulins to the various components of the HBAg could be expected to convey important information pertinent to the pathogenesis of hepatitis and the carrier state.

The normal immune system seems to respond vigorously to the virus and the result may be severe, even fatal, hepato-cellular destruction, whereas a reduced immune response or state of immunosuppression results in mild clinical disease (Sherlock, 1976). The humoral response to HB virus infection may be used to measure the immune reaction to at least the presently known antigenic determinants of the hepatitis B antigen. The surface antigen is antigenically complex and there are multiple specificities within this antigenic system (Levene and Blumberg, 1969; le Bouvier, 1971). A group-specific determinant a is present in all HBsAg positive sera and two pairs of mutually exclusive sub-determinants, d or y and w or r seem to represent virus sub-types. The antigenic types appear to breed true, that is the index and secondary cases may be shown to possess identical sub-types, and this indirect evidence indicates that the sub-types are phenotypic expressions of viral genomes rather than antigen variations determined by the host (Reviewed by Robinson and Lutwick, 1976).

Distinct from this system and of importance in the pathogenesis of the disease, is the e-antigen and its antibodies. This family was detected by Magnius and

Espmark (1972), who noted the appearance of precipitin lines in a double diffusion agarose gel when HB antigen positive serum from renal dialysis patients was placed adjacent to HB antigen positive serum from a multiply transfused haemophiliac. Further characterization of this system (Magnius, 1975), once it had been shown that the e-system was associated with type B infection (Eleftheriou et al., 1975), showed that it differed from HBAg in essential physico-chemical properties. In particular, the molecular weight of 300 000 daltons and a sedimentation coefficient (S) of 11.3 indicated that Magnius was investigating a soluble antigen of intermediate globulin dimension and not an antigen on the surface of HBAg particles. Both el Sheikh et al., (1975) and Nielsen et al., (1974) found a high correlation between Dane particles in sera and e-antigen and Nordenfeldt et al., (1975) showed an association between this antigen and DNA polymerase activity. During the investigation of 70 HBAg carriers, Feinman et al., (1975) found that e-antigen was associated with abnormal liver biopsies characterised by portal infiltrates and that e-antigen occurred in those carriers who had normal liver biopsies or less severe forms of hepatitis. In a small series of chimpanzee and human cases of hepatitis, e-antigen was associated with nuclear core antigen; those cases with definite anti-e did not reveal core antigen in hepatocyte nuclei in spite of the demonstration of HBs antigen in the cytoplasm in five out of six biopsies (Murphy et al., 1976). The authors admit that the biopsies may not have been truly represent-

ative. The e-antigen has been detected early in acute hepatitis, even during the incubation of the disease (Magnius et al., 1975; Nielsen et al., 1974) but was not detected in 99 cases of acute hepatitis with no sequelae, although 14% of these cases developed anti-e. Persistence of e-antigen indicates a more serious prognosis and up to 44% of cases of chronic active disease have detectable e-antigen in the serum (Eleftheriou et al., (1975). Similar findings were reported by Nielsen et al., (1974) and Fay et al., (1975). Vogten et al., (1976) associated e-antigen with failure to clear HBsAg and to respond to treatment with steroids.

The increased infectivity of e-positive HBsAg is also indicated in a study compiled by Grady (1976) in which transmission of infection following accidental inoculation of medical personnel was twice as high when e-antigen was detected in the contaminating blood. This incidence should not be considered alone, as other aetiological factors may be present in the series, especially as the study included dialysis staff. It was also shown that the incidence of e-antigen amongst HBAg positive dialysis patients was considerably higher (76%) than in other areas (28%).

In an important study, stimulated by the observation that HBAg positive mothers could be separated into two groups by assessing vertical transmission of the e-antigen, Okada et al., (1976) screened 23 mothers for the

presence of e-antigen and e-antibody. All the babies born to e-antigen positive mothers (10/23) were HBsAg positive, as were elder siblings; the e-antigen negative mothers (7/23) did not transmit the HB antigen to their babies. In addition, all maternal sera with e-antigen contained Dane particles which were not detected in those sera which had antibody to e.

The antibodies of the e family may be considered evidence of an immunological response to e-antigen, a soluble, non-particulate antigen occurring in sera which show evidence of hepatitis B infection in that they are infective, contain Dane particles and are associated with active liver disease. In acute hepatitis, e-antibody occurred as early as the fifth week after the onset of jaundice in a small series reported by Eleftheriou et al., (1975), and before the disappearance of HBAg. The occurrence and persistence of e-antibody in a wider sense remains largely unprobed and awaits the development of a more sensitive screening technique.

Recently a link between the soluble antigen of Magnius and the morphological forms of hepatitis B antigen has been described. Neurath et al., (1976), using affinity chromatography, purified antibody to e-antigen. Immune electron microscopy showed that the e-reactive site was present on the Dane particles and on the filamentous forms of the surface antigen.

Neurath and Strick, (1977) in a letter to the Lancet, have recently amended this view and indicate that e may be isolated by insolubilised antibodies to human IgG (but not anti-IgM or antibodies to non-immunoglobulin serum proteins). They suggest that e-positive molecules, eluted from the immunosorbent, are dimers (or higher polymers) of a 7S immunoglobulin and may be anti-antibody. Experimental details are not given, nor is the amendment to the earlier publication clear in respect of the isolation of e-determinant Dane particles and filaments. The physicochemical properties of the e-positive molecules would now seem to be similar to those characterised by Magnius (1975).

It is apparent from the literature that the e family of antigens consists of at least two sets of reactants (Magnius, 1975; McAuliffe et al., 1976) and cross reactions are possible. The significance of this family may be of great use clinically (Sherlock, 1976) as the presence of e-antigen may be used prognostically to determine continued viral replication and Dane particle production.

Chapter 2

POLYMER PRECIPITATION

INTRODUCTION

In the light of the frequent application of polyethylene glycol (PEG) to a wide variety of fractionation procedures in this laboratory, it was decided to investigate the possibility of using this polymer for the isolation and concentration of HB antigen from human serum. In the absence of other sources of this antigen, except in very rare instances, it was most essential to have a standard procedure for preparing HBAg in large quantities and in a suitably purified state from plasma, for future experiments.

Selective precipitation by PEG of proteins (Polson et al., 1964) of viruses (Polson and Deeks, 1963) and of bacteriophages (Yamamoto et al., 1970) is now commonly used as one of the steps in fractionation procedures of complex mixtures.

The use of the polymer is empirical and conditions must be experimentally determined for each system. Juckes (1971) has defined some of the parameters concerned, based on the concept that the process is one of colloid phase separation. Polson (1972) has developed the theory that the protein is displaced from solution by super-

concentration in the inter-PEG spaces.

The solubility of the protein either alone or in a mixture of proteins such as plasma, would seem to depend on a concentration of the protein(s), the ionic strength and pH of the mixture, as well as the size of the protein molecule. To establish the parameters and to standardise the fractionation procedure, variations in the preparation pattern may be minimized by diluting the plasma with Sörensen's buffer thus ensuring a relatively constant pH and protein concentration. At pH 8.6 the charge difference between albumin and globulin increases and this has been shown by Polson and Parker (1973) not only to enhance the separation of these two fractions but to render the albumin free of HB antigen.

The parameters for the fractionation of HBAg from serum by polymer precipitation were unknown at the time of the initial experiments in the present study, but since it was important to have a ready method for procuring concentrated HBAg from serum a standard procedure using PEG was developed. High levels of purity at this stage were not critical but in the processes of concentration and separation, retention of morphological integrity of the Dane particles was essential.

METHODS

The procedures evolved in this study are described below. Pulverised PEG was used in Method 1a; PEG in solution was used in Method 1b; Method 2 is an example of differential precipitation obtained by altering the pH of the solution while the PEG concentration remains constant.

METHØD 1a. Fractionation with pulverised polymer (PEG)

Polyethylene glycol m.w. 6 000 was pulverised in a mortar until smooth, or in a Waring blender for the rapid preparation of bulk amounts.

The plasma to be fractionated was diluted 1:3 with Sörensen's buffer pH 8.2 and the pH checked and adjusted to pH 8.6 with crystals of $Na_3PO_4 \cdot 12H_2O$.

PEG 5% w/v was slowly added to the diluted plasma which was kept briskly stirred by a magnetic stirrer, until the PEG was completely dissolved. The mixture was allowed to stand at room temperature to allow phase separation to reach completion, and the precipitate removed from the mixture by low speed centrifugation at 1 000 x g 20 min. The supernatant fluid was decanted into a measuring cylinder and the new volume determined. The concentration of PEG in the supernatant could then be calculated from the formula

$$V_1C_1 = V_2C_2$$

where V_1 is the volume in ml of the original diluted serum and C_1 the concentration of PEG in gm per 100 ml. V_2 is the volume of the SNF and C_2 is the new concentration of PEG in gm per 100 ml. Pulverised PEG is added in the same manner to bring the concentration in the supernatant up to 20% and the resultant precipitate is packed by low speed centrifugation. The supernatant fluid was carefully discarded. The surface of the sedimented pellet and the walls of the centrifuge tube were gently rinsed with distilled water to reduce contamination of the pellet by PEG. A volume of modified Sörensen's buffer pH 8.6 equal to the original plasma was delivered into the centrifuge tube and the pellet dissolved in situ with the aid of a small magnetic stirrer. This was a solution of what was designated for convenience the "20% PEG precipitate."

In order to ascertain the advantages of re-precipitation of this product, the dissolved 20% PEG precipitate was subjected to further fractionations by sequential additions of more PEG. The cycle was commenced by the addition of 2% w/v of PEG to the solution, the removal of the precipitate, the determination of the volume of the supernatant, and the recalculation of the amount of PEG in the supernatant. The concentration of the polymer was then increased with the addition of 2% PEG with each step in the process until a 10% concentration was reached. The last increment was of 5% PEG to give a final concentration of 15% PEG. These precipitates were designated

"2% to 15% PEG re-precipitates" and each was separately dissolved in phosphate buffer pH 7.2 in a volume equal to 1:5 of the original plasma volume.

METHOD 1b. Fractionation with a 30% solution of PEG

Another procedure that was investigated made use of a 30% solution of PEG. The volume of this solution required to give a particular final concentration of PEG was calculated from the formula

$$C_1V_1 = C(V_0 + V_1)$$

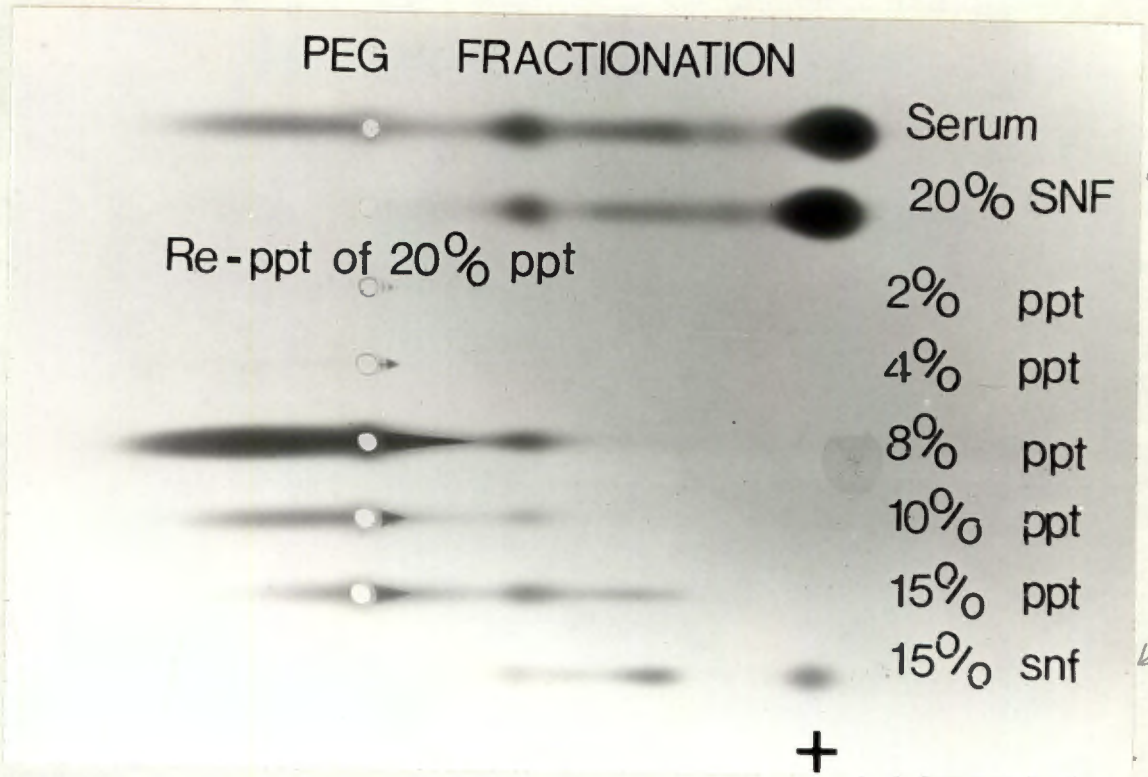
where C_1 is the concentration of PEG in gm per 100 ml; V_1, the volume of PEG solution in ml; V_0, the volume in ml of the mixture to be fractionated, and C, the required final concentration of PEG in gm per 100 ml.

Example: To determine the volume of 30% PEG solution that must be added to 25 ml of diluted serum to give a PEG concentration of 10% (gm per 100 ml).

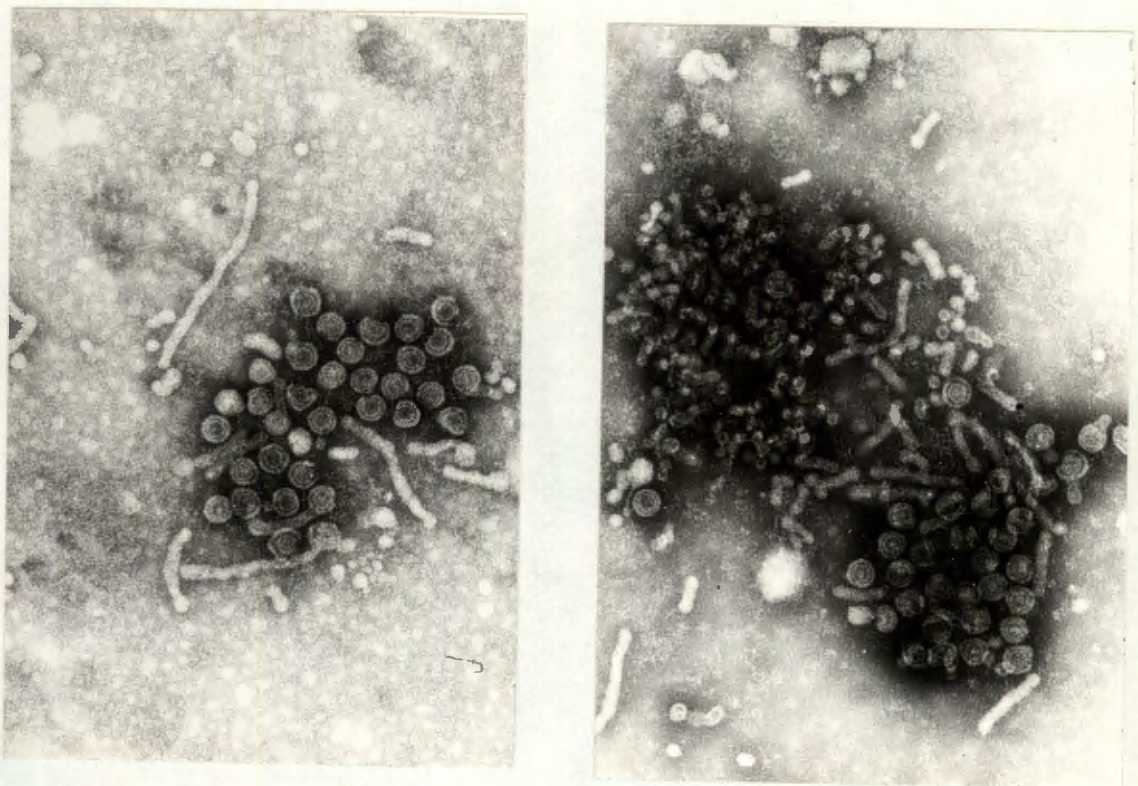
$$30V_1 = 10(25 + V_1)$$
$$30V_1 = 250 + 10V_1$$
$$20V_1 = 250$$
$$V_1 = 12.5 \text{ ml of the 30\% PEG solution}$$

METHOD 2. Fractionation with pulverised PEG and pH shift

The 20% PEG precipitate from Method 1a was diluted with phosphate buffer pH 3.5 in a ratio of 1:3 and the pH of the mixture adjusted to 3.5 with 1N HCl.



2.1.a. Gel electrophoresis in 1% agarose (pH 8.6) of fractions displaced by incremental precipitation with PEG. Precipitates dissolved in equal volumes of buffer equal to 1/5 volume of serum.



2.1.b. Complexed HBAg precipitated from dilute solution of the "20% PEG precipitate" by reprecipitation with 2% PEG w/v. Magnification x 80 000

PLATE 2.1.

While stirring, pulverised PEG was slowly added to the mixture to provide a concentration of 7% w/v PEG. The fine precipitate that formed was removed by low speed centrifugation and the pH of the mixture raised to 4.2 by adding crystalline Na_3PO_4. The resulting heavy precipitate was removed and dissolved in phosphate buffer at pH 7.2.

RESULTS

METHOD 1.

The plasma fractions, precipitates and supernatants, produced by the different concentrations of the polymer were evaluated by electrophoresis in agar gel or on cellulose acetate and also examined in the electron microscope for the presence or absence of hepatitis B antigen.

The initial precipitation with PEG removed visible impurities from the plasma, including residual cell debris. Electrophoresis of this fraction showed a single peak of precipitated protein in the position of fibrinogen. Only a trace of antigen was present in this fraction.

Electrophoresis of the "20% PEG precipitate" showed that a separation of the remaining proteins had been achieved but with some overlapping on either side of a line centred about the region of the alpha globulins. The "20% SNF" (Plate 2.1.a., Fig.2.1.a.) contained none of the gamma but most of the albumin with the faster migrating alpha

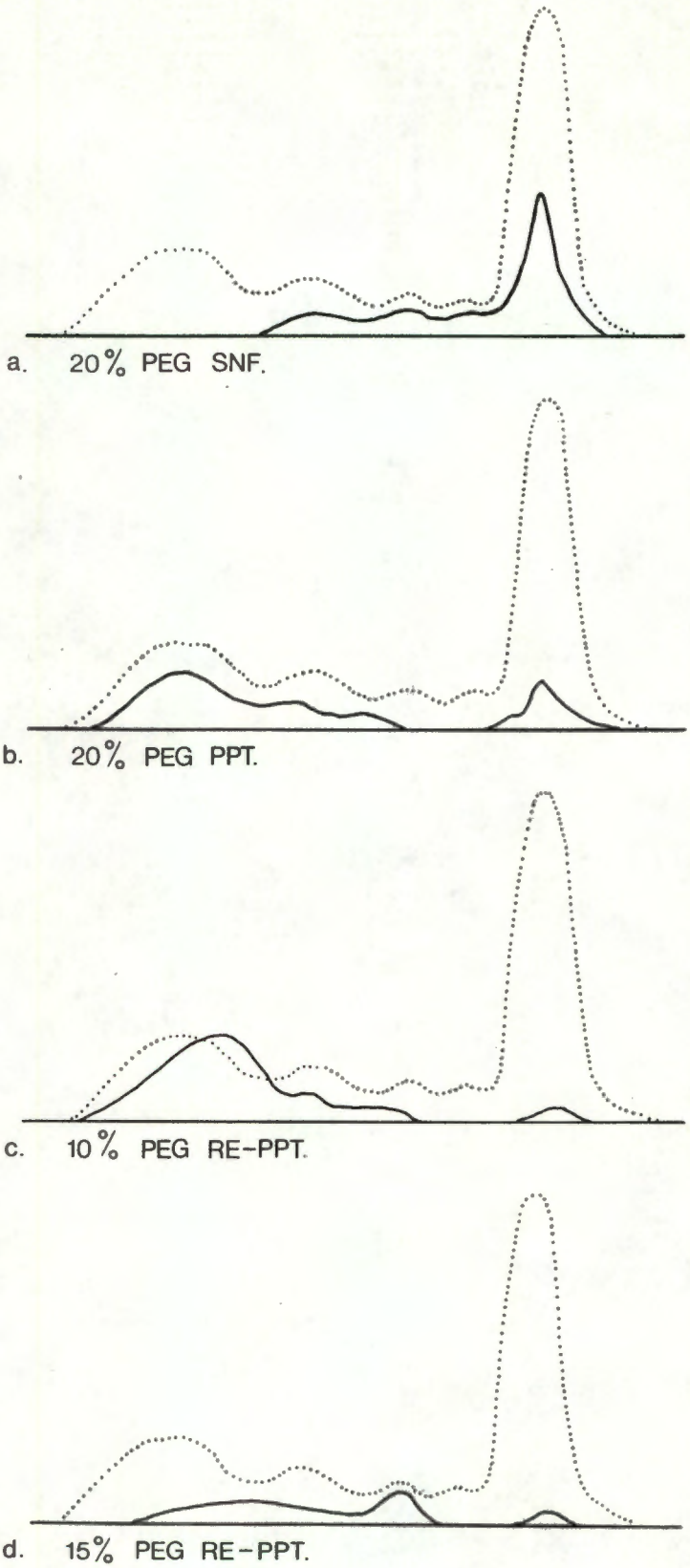


FIG. 2.1. CELLULOSE ACETATE ELECTROPHORESIS

and beta globulins. The "20% PEG precipitate" (Fig.2.1.b.) had most of the gamma globulins with the alpha and beta globulins and a small amount of the albumin.

Re-precipitation of the "20% PEG precipitate" with 10% and 15% PEG produced a displacement of the gamma globulins and some of the other globulins at 10% PEG concentration (the "10% PEG re-precipitate") (Fig. 2.1.c., Plate 2.1.a.) followed by most of the remaining globulins present in the fraction at "15% PEG re-precipitate" (Fig. 2.1.d., Plate 2.1.a.)

Observations on the effects of sequential 2% increments of PEG added to the solution of the "20% PEG precipitate" are presented in Plate 2.1.a. The serum fractions found in the resulting precipitates varied markedly. This procedure made it possible to decide on the most appropriate treatment for the isolation of the HB components.

The flow-chart, Table 1, lists the steps of the two Methods 1 and 2. Each precipitate and each supernatant was examined by electron microscopy for the presence of HB antigen. Many of them gave unrewarding yields of one or other of the components of HB antigen, but the results of the most successful isolations are positioned alongside the relevant fraction. Complexed HBAg was removed by re-precipitation with 2% PEG (Plate 2.1.b.). It will be seen that the remaining HB antigen components were represented

TABLE 2.1.

	Distribution of HB Antigen & globulins		
	Dane particles & filaments	Small spheres	Gamma globulin
METHOD 1			
1 vol serum + 2 vols buffer pH 8.2			
+			
Na_3PO_4 → pH 8.6 + 5% PEG w/v			
→ PPT	0	±	Fibrinogen
SNF			
to			
20% PEG w/v			
"20% SNF" ←			
"20% PEG precipitate"	++++	++++	++++
+			
1 vol buffer pH 8.6			
+			
2% PEG w/v			
→ "2% PEG re-ppt"	Complexed HBAg		0
SNF to 10% PEG			
→ "10% PEG re-ppt"	+++	++	+++
SNF to 15% PEG			
→ "15% PEG re-ppt"	0	+++	+
SNF			
METHOD 2			
"20% PEG precipitate"			
+			
Buffer pH 3.5			
+			
7% PEG w/v			
→ "7% PEG pH 3.5 ppt"	0	++++	0
SNF			
→ pH 4.2			
→ "7 % PEG pH 4.2 ppt"	0	0	++++
SNF			

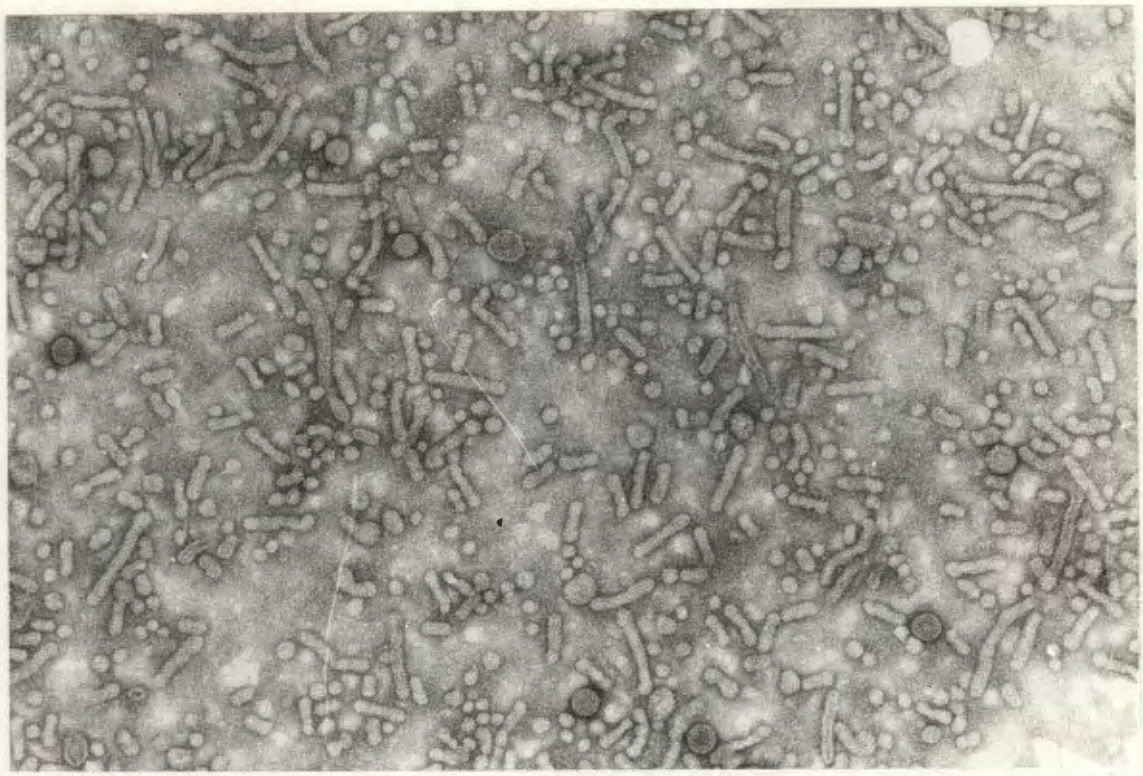
by two distinct populations. The "10% PEG re-precipitate" contained apparently all the Dane particles, most of the filamentous forms and a proportion of the small spheres (Plate 2.2.a.). The "15% PEG precipitate" contained an almost homogeneous population of small spheres with occasional short filamentous forms not longer than twice the diameter of the spheres (Plate 2.2.b.).

The number of Dane particles and filamentous forms increased with each 2% increment of PEG, and the maximum yields were with the 8% and 10% concentrations of the polymer.

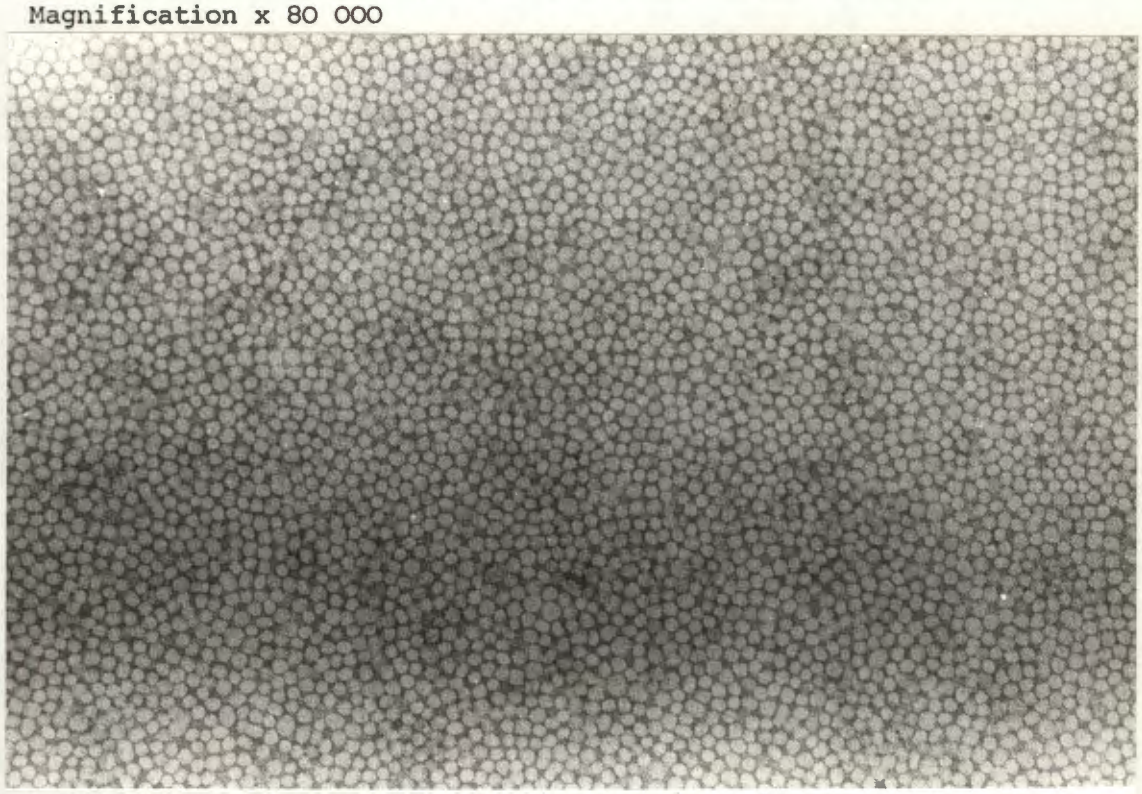
METHOD 2.

The precipitate produced by re-precipitation of the "20% PEG precipitate" at pH 3.5 with 7% PEG was found to contain no detectable serum proteins by electrophoresis on cellulose acetate or by immuno-electrophoresis in agarose gel using anti-human serum prepared in rabbits. Shifting the pH to 4.2 without altering the PEG concentration resulted in the formation of heavy precipitate which, on electrophoresis, proved to consist almost entirely of globulins.

On electron microscopy the HB antigen was present only in the initial precipitate formed at pH 3.5. None of the antigen appeared in the precipitate at pH 4.2. In the former, the HB antigen consisted of small spheres only and



2.2.a. Large component HBAg displaced by re-precipitation with 10% PEG from the dilute solution of the "20% PEG precipitate". Magnification x 80 000



2.2.b. Small spherical antigen displaced from the SNF of the 10% PEG re-precipitation of the "20% PEG precipitate" by increasing the concentration of PEG to 15% w/v. Magnification x 80 000

PLATE 2.2.

was not associated with detectable serum proteins. Although the surface structure of the antigen appeared to have lost some detail, one dimensional Laurell electrophoresis in a gel containing anti-HBs confirmed the high concentration of HBs antigen in the pH 3.5 fraction and absence of HBs antigen in the fraction produced at pH 4.2.

The influence of temperature on these fractionation procedures was noted in the initial experiments. No appreciable difference was observed when the procedures were carried out in parallel on the same sera, one at 4°C and the other at room temperature of 22°C. All fractionations were thereafter performed at ambient room temperature.

DISCUSSION

The removal of fibrinogen from plasma with 5% PEG w/v had the additional advantage of clarifying the serum by precipitating cell fragments and denatured lipoproteins present in some of the plasmas which had been stored for long periods of time. The loss of very small amounts of HB antigen in the precipitate was presumed to be as a result of co-precipitation by the mass of precipitated fibrinogen.

The most optimistic anticipations at the commencement of these investigations were that PEG would achieve

one or more of several goals. It was thought that if due attention were paid to the parameters known to influence the outcome of the fractionation procedures, such as the protein concentration of the mixture, the pH, the ionic strength and the polymer concentration, it might be possible

i. to separate and concentrate the components of HB antigen in the serum sample

ii. to separate and concentrate the gamma globulins in the serum

iii. to separate i from ii.

These expectations were not fully realized but the attainments and success were such as to be of inestimable value in the subsequent investigations.

On the assumption that a relatively high concentration of serum proteins and individual variations in lipoprotein content might adversely influence the displacement of the HB antigen by PEG, the serum was regularly diluted with buffer. This step was shown to be important and this was confirmed by many fractionations of sera from different patients in the routine preparation of HB antigen for investigation.

The steps in the fractionation procedure listed in Table 1 represent a selection of the very many variations

of polymer concentration and conditions of pH and ionic strength that were tried in attempts to produce the optimum results. Even using very small increments in PEG concentration failed to select a set of conditions where separation of HB antigen from serum proteins was complete. It is obvious however that the "10% PEG re-precipitate" gave the most worthwhile results, and this was used as the standard method of preparation of antigen thereafter, when Dane particles were required and the "15% PEG re-precipitate" when small spheres alone were needed.

Although this proved to be a useful division between the morphological forms of the antigen, it was clearly not one that depended solely on particle size. Antigen which remained in solution after 10% PEG precipitation, consisted almost entirely of small spheres of very uniform shape, while the precipitated antigen contained all the Dane particles, the filamentous forms (longer than 2 sphere diameters) and approximately 1/3 of the small spheres.

An attempt was made to correlate the mean diameter of the "insoluble" small spheres with that of the "soluble" small spheres at this concentration of PEG. Electron micrographs of these two fractions were assessed using a micro-comparator. A hundred spheres of each of these samples were measured using arbitrary units and the means of the two diameters compared. No correlation was detected in size and it was presumed that other factors

such as surface charge density, govern the separation of the small spheres at this intermediate point. A similar finding was reported by Neurath et al., (1973a) at neutral pH and under different ionic conditions.

The small spheres precipitated by 15% PEG had the advantage that serum proteins were markedly reduced and that it was relatively free of antibody globulins. On the other hand the use of the large component fraction precipitated by 10% PEG was complicated by the co-precipitation of immunoglobulins which frequently included antibodies to the HB core or other cryptic antigens.

The presence of HB antigen and the appropriate HB antibody results in the formation of antigen-antibody complexes under certain conditions. Small numbers of complexes in serum samples containing large amounts of HB antigen may be very difficult to detect in the electron microscope, but by re-precipitation of the samples with 2% PEG, complexed antigen of the larger classes were displaced without rendering Dane particles and the larger filaments insoluble. Such small increments of PEG were selectively used to detect immune complexes when present in small numbers.

Method 1 therefore provided a preparative technique for the concentration of semi-purified antigen from plasma. The one disadvantage of this method was the co-precipitation of immunoglobulins with the large components

of the HB antigen. For many subsequent investigations it was essential that the immunoglobulins be separated from the antigen, particularly when it became obvious that most of the donors, who had become carriers of HB antigen, had in their serum an antibody directed to an inner component of the Dane particle. Nevertheless this method quickly and easily provided concentrated antigen in two population sizes, if necessary from large volumes of plasma, but with the large component fraction always associated with immunoglobulin. To separate these two components by polymer precipitation an additional step was introduced. The isoelectric point (pI) of the macroglobulins is centred at pH 6.0; that of the heterogeneous antigen extends from pH 4.5 - 5.4 in unfractionated samples, to pH 3.9 - 4.9 for purified antigen (Chairez et al., 1975). This difference in solubility at these pH points allowed polymer precipitation linked with pH shift to be used. When the concentration of the polymer was kept constant at a level which would induce displacement only in conditions of minimal solubility, that is within one pH unit of the pI of the protein, the pH was shifted sequentially to allow the differential exclusion and separation of these components (Johnson and Newman, 1972 and 1974).

Method 2, based on this hypothesis, utilized the first step of the precipitation of HB antigen and its globulins by 20% PEG. The precipitate was dissolved in buffer at pH 3.5 and 7% PEG was added. As judged by quantitative Laurell electrophoresis with anti-HBs in the

gel, all the HB antigen components were precipitated. On shifting the pH to 4.2 with the PEG concentration remaining at 7% w/v, a heavy precipitate was produced which was shown to consist of globulins. This procedure thus separated HB antigen from the immunoglobulins present in the donor's serum. On electron microscopy the preparation was found to consist of small spheres only, in spite of the presence of an abundance of the large components in the original serum. As the purpose of the fractionation was to provide a source of immunoglobulin-free Dane particles for serological study, the loss of these particles rendered the method unsuitable for immune electron microscopy.

The retention of HBs reactivity of the damaged antigen is in keeping with the findings of Dreesman et al., (1972) who treated Dane containing HBAg at pH 2.4 for 1 hour and recorded the loss of large components from the purified HBAg. This preparation was used to determine the molecular weights and isoelectric points of hepatitis B. Antigenic activity was retained and antiserum raised in guinea pigs did not show any activity against normal human serum components.

Nath et al., (1976) have recently described a procedure for obtaining HBAg from plasma by precipitation with PEG 4 000. This PEG was added to undiluted plasma to a concentration of 8% w/v and the precipitate was removed and discarded before the concentration in the SNF was

raised to 16%. The precipitate produced by this increment of PEG contains the HBsAg, which was then digested with pepsin at pH 2.3 and treated with Tween 80. The antigen so prepared was not examined in the electron microscope, but was found to have a molecular weight of greater than 100 000 daltons and to be suitably pure for serological use.

Exposure to low pH, or detergent (Tween 80 and Mucasol) has an easily discerned effect on the morphology of the antigen, the Dane particle in particular, but this does not destroy the antigenic integrity of the HBsAg (Gerin et al., 1969; Chairez et al., 1975; Stannard and Moodie, 1976). The pH shift described in Method 2, although damaging to Dane particles and filaments, was shown to be suited to the preparation of HBsAg for serological procedures.

The recent observations by Nath et al., (1976) though differing in detail from the procedures developed in the course of the present study, gives clear confirmation of the necessity for the rather complex preparative procedures to obtain a suitable preparation of hepatitis B antigen.

Chapter 3

THE PREPARATION OF DANE PARTICLES AND THE CORE ANTIGEN

INTRODUCTION

The importance of the Dane particle and the growing need to investigate some of the antigenic properties of its coat and its core, required preparations for electron microscopy which were both rich in Dane particles and free from iso-antibodies. The inner core antigen (HBcAg) was known to have a determinant separate from the surface antigen (HBsAg) (Almeida et al., 1971) and it was suspected that antibody to this component was commonly present in Dane particle containing sera.

The antigen-antibody complexes reported in the sera of chronic carriers (Stannard et al., 1973) could largely be removed by the 2% PEG re-precipitation step (Chapter 2) before concentration of the large component antigen of Dane particles and filaments. This provided a preparation of HB antigen free of complexes that could be used for the detection of antibodies by immune electron microscopy. The presence of the antibody would then be manifest by the formation of new complexes with the homologous antigen.

Simple separation of the antigen in diluted serum by high speed centrifugation followed by two washes in phosphate buffer was time consuming and produced a low

yield of antibody-free Dane particles. Hoofnagle et al., (1973) using HBcAg obtained from chimpanzee livers, confirmed that all Dane particle-containing human sera possessed anti-HBc activity and also demonstrated the presence of this antibody by complement fixation in the sera of all chronic carriers tested.

The presence of anti-HBc in the serum of a carrier could be used as a test for the effective removal of antibody globulins from Dane particle preparations. Treatment of the preparation with a suitable detergent (e.g. 2% Mucasol) to release the cores of the Dane particles, provided a precise and sensitive reagent for the detection by immune electron microscopy of anti-HBc by the resultant formation of core complexes.

This permitted the critical evaluation of two simple additional methods for producing globulin-free large component antigen suspensions. The first of these procedures (Section 3.2.) is based on the size differential between HBsAg and the globulins, and the second (Section 3.3.) exploited the difference in net surface charge.

METHODS

3.1. Differential Ultracentrifugation

An aliquot of 0.5 ml of plasma or serum was diluted with 0.06 M phosphate buffer pH 7.2 in a cellulose nitrate tube (1.25 x 5.0 cm) of the Beckman SW 50 rotor. The diluted serum was clarified by centrifugation at 12 000 x g for 10 min and the supernatant fluid re-centrifuged at 50 000 x g for 60 min. The sedimented pellet was thoroughly dispersed in 0.5 ml of the buffer before the tube was refilled with phosphate buffer, inverted once to ensure mixing, and re-centrifuged at 50 000 x g for 60 min. The wash was repeated before the tube was inverted, and allowed to drain. The pellet was suspended either in one drop of distilled water before staining with 2% phosphotungstic acid for electron microscopy, or alternatively in 0.25 ml PBS for serological reactions before preparation for immune electron microscopy.

3.2. Gel Filtration

Commercially prepared agarose beads (Pharmacia Fine Chemicals AB, Uppsala, Sweden) were diluted with phosphate buffer pH 7.2 to an easily workable consistency, de-aerated and packed, with stirring, to a bed height of 35 cm in a 2.5 cm chromatography column. The flow rate was controlled by a sigmoid pump and the eluate was monitored by

u-v absorption at 280 nm. The void volume was determined using the haemocyanin molecule (mol wt 7.9 x 10^6 daltons) of the mollusc Burnupena cincta, the common whelk (Polson et al., 1970). Samples of 2 ml each were applied under buffer on to the gel surface with the pump switched off. To facilitate this the density of the sample was increased by the addition of sucrose crystals (approx. 5% w/v). After stabilization of the applied sample, as shown by the development of a sharp buffer/sample interface, the pump was re-started at a flow rate of 12.5 ml/h. Fractions were collected in 1 ml amounts to each of which 1.0 ml of 4% Mucasol was added and the mixture stored overnight at 4°C. Next day the 2% Mucasol mixture was incubated at 37°C for 1 h before being prepared for electron microscopy.

3.3. Ion Exchange on Formalin Tanned Gelatin Granules

Hepatitis B antigen containing serum was dialysed against three changes of 20 times its original volume of 0.005 M phosphate buffer pH 5.5 at 4°C, each change occurring at approximately 12 h intervals. After dialysis the pH was checked to be 5.5 and the 'euglobulin' precipitate removed by low speed centrifugation.

Bed material consisting of packed 6% formalin tanned gelatin granules (Polson and Katz, 1968), approximately four times the volume of the original serum, was equilibrated with buffer and drained without suction in a

Buchner funnel. The dialysed serum was added to the gelatine granules and the mixture gently stirred with a wooden spatula for 10 min. Mild negative pressure was applied to the Buchner funnel and the fluid which drained from the gel was collected. The gel was washed with one volume of 0.005 M phosphate buffer pH 5.5 and the washing added to the previous fluid yield. The pH was adjusted to 8.6 with crystalline Na_3PO_4 before small increments of pulverised PEG were added, with stirring, to a concentration of 15% w/v.

The solution was allowed to stand at room temperature for 40 min before the fine excluded material was removed by centrifugation at 20 000 x g for 20 min. This material was dissolved in a small volume of 0.06 M phosphate buffer pH 7.2 and all insoluble material removed by centrifugation at 12 000 x g for 10 min.

RESULTS

3.4. Differential Centrifugation

Antigen prepared by differential ultra-centrifugation was relatively free of serum proteins. Its suitability as a core antigen for electron microscopy (i.e. the absence of core antibody, anti-HBc) was shown simply by incubating the 2% Mucasol-treated-antigen at 37°C before screening the preparation for the presence of core complexes. Such antigen preparations now had no anti-HBc

and were therefore free of immune complexes.

The control for this procedure was provided by overnight treatment of whole serum known to contain anti-HBc, by 2% Mucasol followed by incubation at 37°C for 1 h. The formation of Dane core complexes is easily established by electron microscopy as shown in Plate 3.1.a.

Gel Filtration

The u-v absorption pattern of the molecularly sieved material depended on the type of the starting material fractionated. An elution diagram of HBAg-positive serum chromatographed on 6% Sepharose beads is shown in Fig. 3.1.a., but since an identical tracing was obtained with HBAg-negative whole serum, no evidence of HBAg was apparent by spectrophotometry. Examination of the fraction from the initial high peak A which was at the void volume and consisted of totally excluded material, showed that it contained the large components of HBAg by electron microscopy. HB antigen was found in diminishing amounts in peaks A, B and half way into C, but the large component antigen was found only in the rise and central third of peak A, whereafter the proportion of large components of antigen dropped and in the latter part of this peak only small spheres were present. Small spheres could also be detected through peak B and into peak C at which point they were scattered and widely separated.

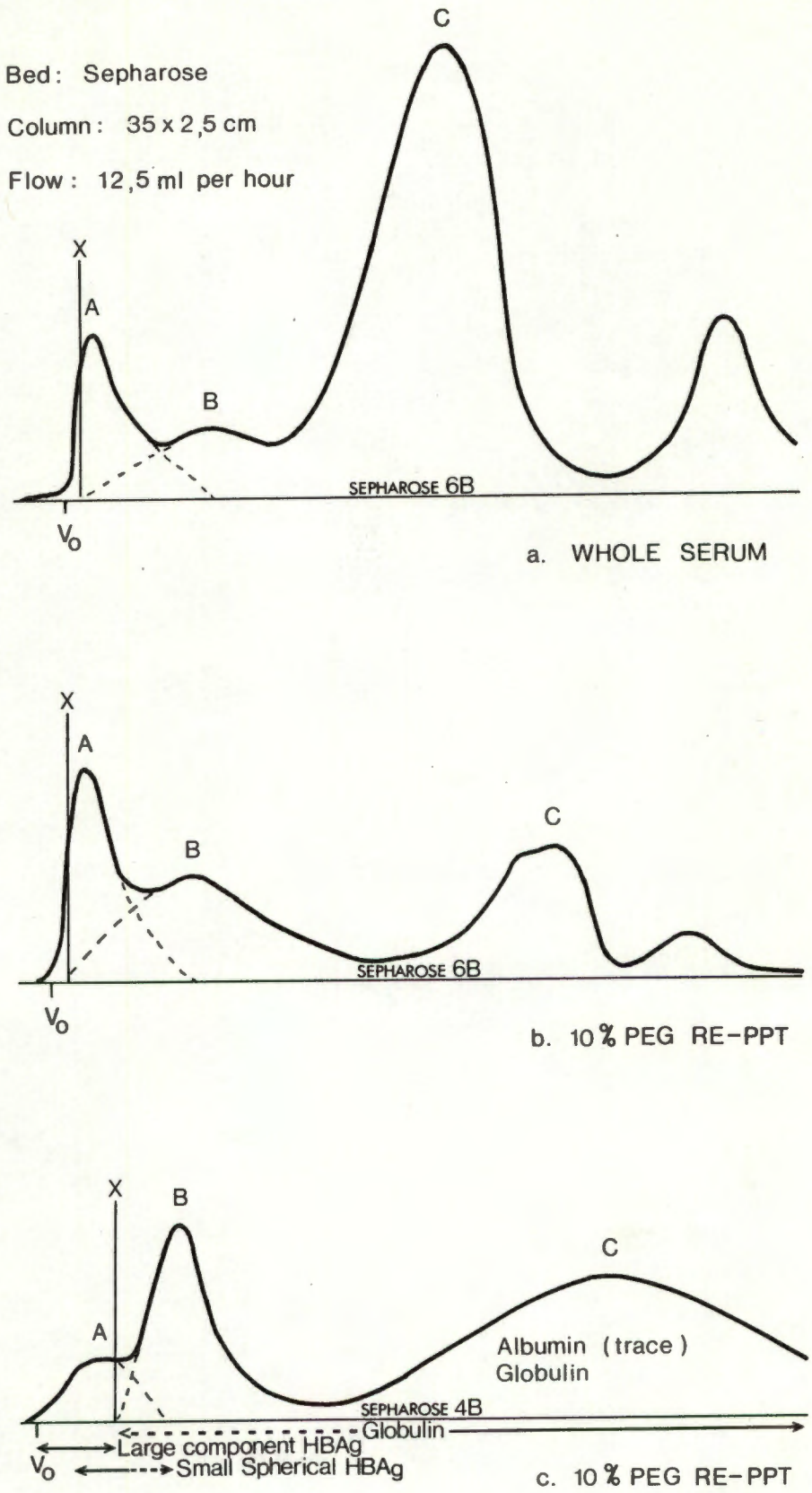
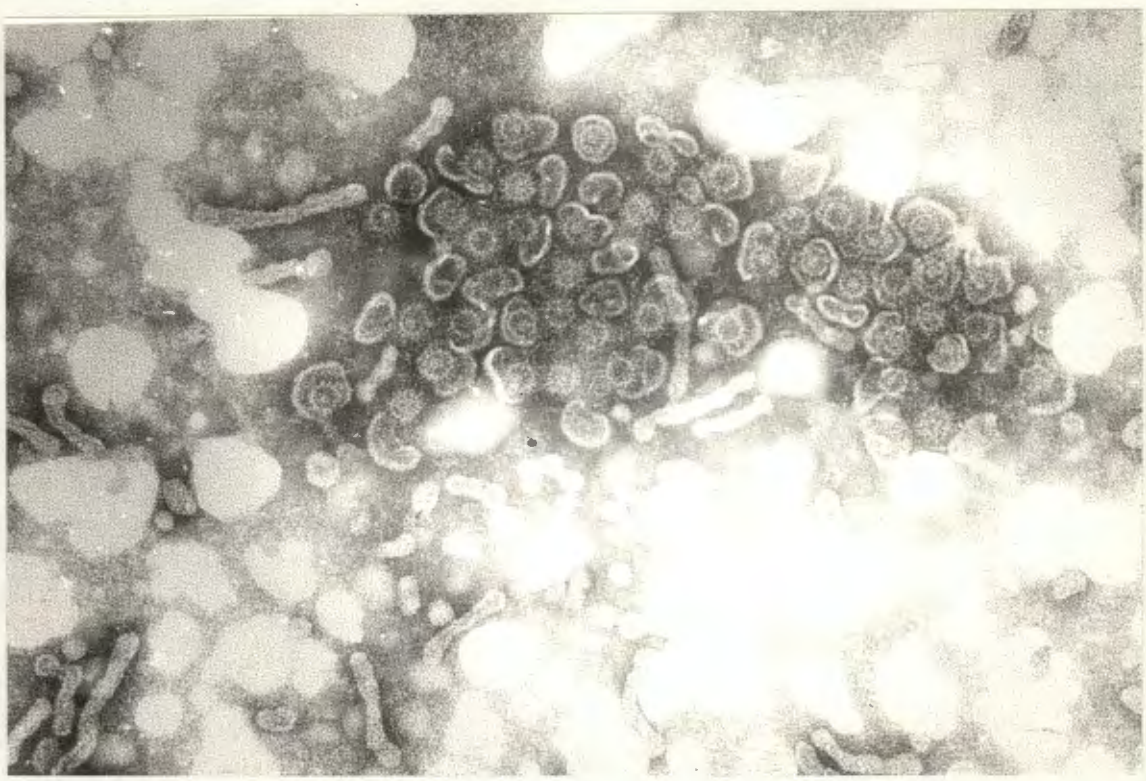


FIG. 3.1. GEL CHROMATOGRAPHY

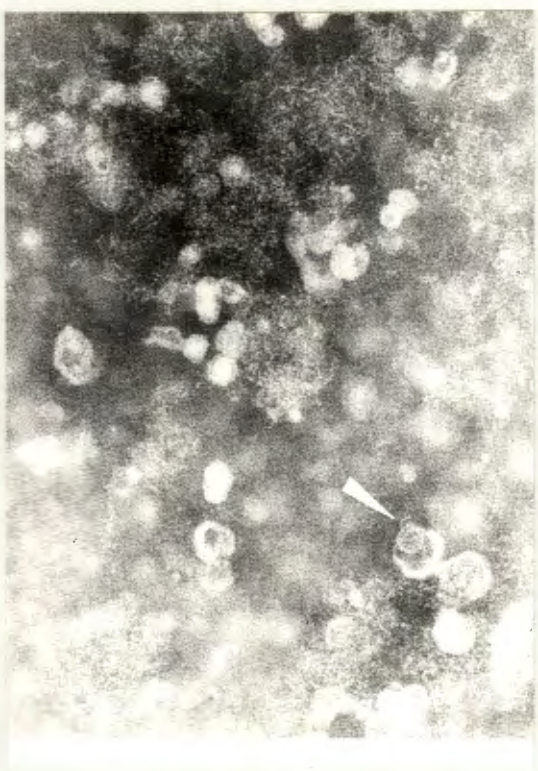
Gel electrophoresis showed that peak C consisted mainly of albumin and that globulins extended from peak B through to peak C.

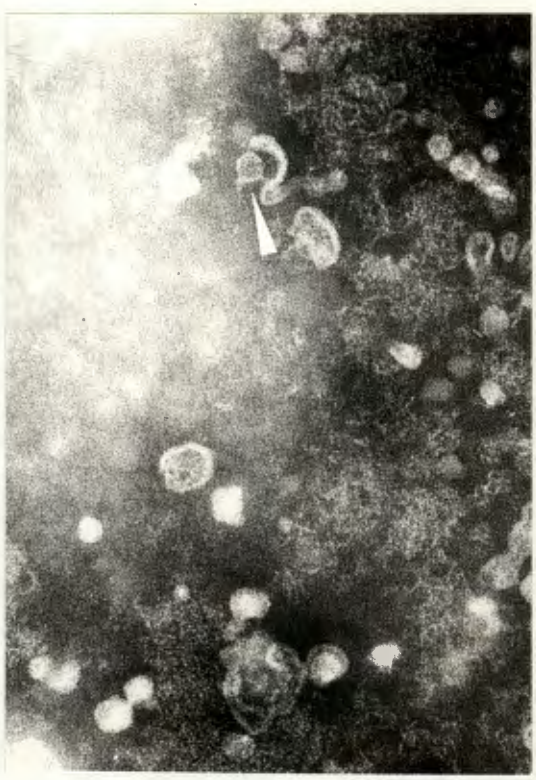
The tracing in Fig. 3.1.b. represents the elution diagram of the 10% PEG re-precipitate fraction on Sepharose 6B. Peaks A and B are again represented as in Fig. 3.1.a. but peak C is much reduced. Electrophoresis showed that most of the material in this peak was gamma globulins with very little albumin. Quantitative assay of this fraction on Tri-Partigen plates after concentration to the original volumes gave values of IgM 32 mg/100 ml and IgG 640 mg/100 ml. Large component antigen was present in peak A and electron microscopic examination after detergent treatment showed that there was no antibody to the released cores.

Using Sepharose 4B instead of 6B the ratios of peak A to peak B changed. Large antigen components were found in peak A. Small spheres were present in peak A and extended into B. Globulins were spread broadly from small amounts in peak B to constitute the major portion of peak C. The appearance of the Dane particles, filaments and small spheres, slightly altered by detergent treatment, is illustrated in Plate 3.1.b. There are no complexes of Dane cores and no anti-HBc.



3.1.a. Detergent treated whole serum. Dane core complexes. Magnification x 120 000





3.1.b. Sepharose 4B fraction from peak A. Detergent treated and incubated. Absence of anti-HBc on cores (arrows). Magnification x 120 000

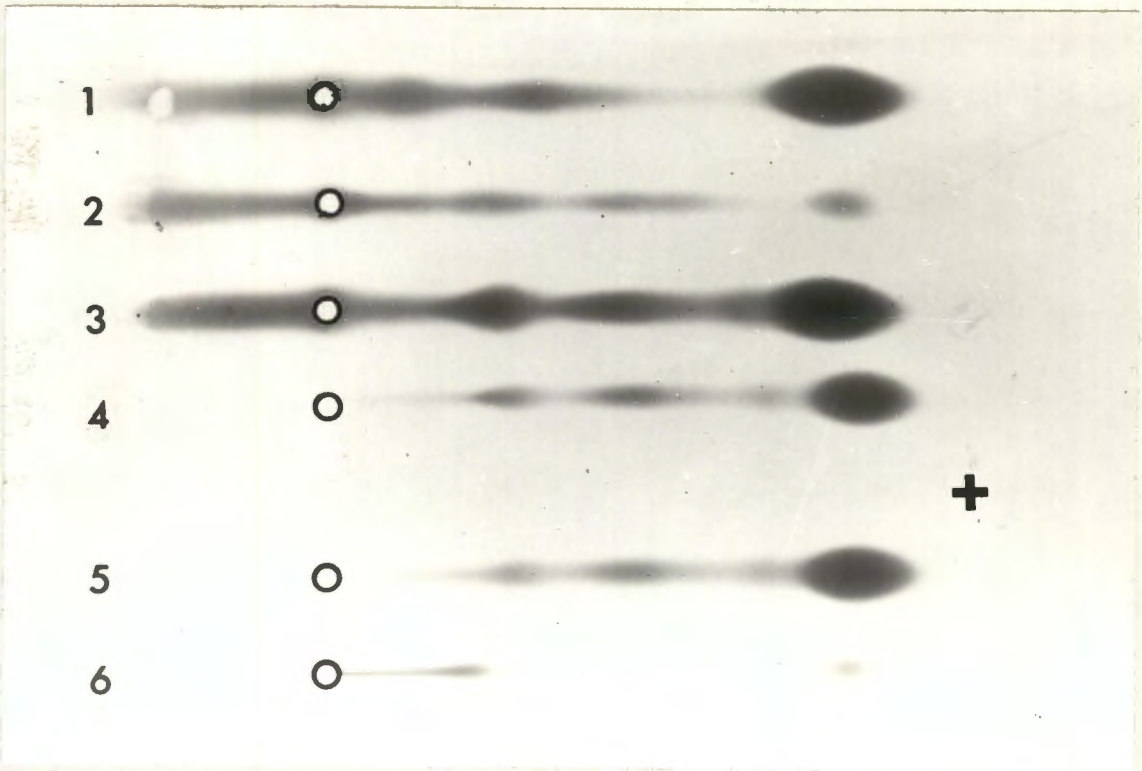
PLATE 3.1.

Ion Exchange on Formalin Tanned Gelatin Granules

The light precipitate formed in the cold when serum was dialysed against low molarity buffer, was dissolved in 0.06 M phosphate buffer pH 8.2 at room temperature and evaluated by electrophoresis in agarose gel before discarding. Gamma globulins together with small amounts of alpha and beta globulins and trace amounts of albumin (Plate 3.2.a.) were detected. On electron microscopy no antigen was detected in this sample.

After cation exchange of the dialysed serum by 6% formalin tanned gelatin granules, the supernatant fluid contained the HB antigen, but no gamma globulins detectable by gel electrophoresis. LC-Partigen plates confirmed the absence of IgG and IgM but showed the presence of a trace of IgA. The buffer used to wash the tanned gelatin contained only a small amount of albumin.

Trial precipitation of the supernatant, with the added buffer wash, by 15% PEG at pH 5.5 showed that under these conditions some of the HB antigen was not precipitated. Precipitation of the same supernatant fluid mixture with the pH increased to 8.6 resulted in the displacement of all the HB antigen by 15% PEG with only small amounts of protein (mainly albumin and β-lipoproteins) detectable by gel electrophoresis. (Plate 3.2.a.7.). Examination of this antigen under the electron microscope showed slight loss of surface structure and



3.2.a. 1% Agarose gel electrophoresis (pH 8.6) of fractions from ion exchange (see Section 3.3.) on formalin tanned 6% gelatin granules pH 5.5

1. HBAg +ve serum diluted 1:5 in buffer pH 8.6
2. Concentrated solution of precipitate formed on dialysis of the serum to molarity 0.005 and pH 5.5.
3. Original serum after dialysis to molarity 0.005, pH 5.5.
4. Post cation exchanged serum pH 5.5.
5. SNF of post cation exchanged serum 15% PEG pH 8.6 HBAg -ve.
6. Solution of 15% PEG precipitate (10 x concentrated) HBAg +ve.

3.2.b. HBAg from 7 (above).

PLATE 3.2.

the majority of the Dane particles were intact. After Mucasol treatment no antibody molecules were to be seen on the exposed cores.

DISCUSSION

Preparing small amounts of HBAg for electron microscopy by ultracentrifugation was the most simple and reliable procedure, but was time-consuming where repetitive screening was required.

Gel filtration is an obvious choice for separating large component antigen especially when concentrated preparations are available and when very large scale fractionations are not needed. The size differentiation between the small antigen with a molecular weight of 2.5×10^6 (Howard, 1975) or $3.7 - 4.6 \times 10^6$ daltons (Chairez et al., 1975) and the globulins (IgM 890 000 daltons; Merler, 1970) should allow separation by molecular sieving. In practice this was achieved and if polymeric or aggregated globulin contaminated the HBAg containing preparation, specific anti-HBc was not detected. Both Burrell et al., (1973) and Howard and Zuckerman, (1973) used Sephadex G 200 for partial purification of antigen from serum proteins, but agarose beads with higher flow rates and quick packing properties were found to be adequate for producing globulin-free large component antigen. Both Sepharose 4B (exclusion limit 20×10^6) which retarded large and small HBAg

(Fig.3.1.c.) and Sepharose 6B (exclusion limit 4 x 10^6) which voided large antigen (Fig.3.1.b.) had the added advantage of partially separating small spherical antigen from the Dane particle containing components.

The starting material used for this separation procedure should be the concentrated large component antigen and the source of such material was available in the re-precipitation fraction of the PEG fractionated material. This 10% re-precipitate could be stored at 4°C and (with the addition of 5% sucrose) layered directly on to the column. The fractions collected immediately after the void volume could be used without further concentration for immune electron microscopy and 0.5 ml of this material was found to be a "grid dose". The term "grid dose" was used to imply the volume of a suspension of antigen which when sedimented and washed, yielded the correct amount of antigen for the evaluation of one procedure by electron microscopy. Too much antigen present in the sample gave rise to overdense particles on the grids and the consequent masking of the antigen/antibody complexes. Too little, although allowing fine resolution of detail, caused practical problems in staining and coating of the grids and sometimes made the addition of bovine serum albumin obligatory in order to produce sufficient surface tension to coat the grid. This emergency measure clouded resolution so that molecules of antibody were lost in the background of the added protein.

The gel filtration method was developed for the production of anti-HBc-free Dane particles for screening sera for the presence of core antibody and for studies on the Dane core and its antibodies. The advantage of this method was the production of batches of semi-purified antigen which could be stored and utilised when necessary. The degree of purity was adequate for immune electron microscopy.

The separation of HBAg from globulin by ion exchange was limited by the effect of low pH on the Dane particles. Examination by electron microscopy of antigen prepared from a series of experiments at varying pH values, showed that exposure of the antigen to pH below 5.2 resulted in a disruption of the Dane coat. In addition changes were noted in the surface structure of the small antigen which under extreme conditions (pH 3.7) became unrecognisable. These changes were similar to those caused by glycine/HCl buffer pH 2.45 described by Hirschman et al., (1973).

Initial experiments using both PEG precipitated fractions and whole serum indicated that a pH value intermediate between that of the iso-electric points of HBsAg, 3.8 - 4.1 (Howard and Zuckerman, 1973) or 3.9 - 5.3 (reviewed by Melnick et al., 1976) and the globulins (pI 6.1 - 6.8) could make use of the opposite charges of these two components to achieve separation. It was found that the upper limit of pH required for the adsorption of all the globulins to the anionic gel was pH 5.2 but at

this pH the large component antigen showed signs of damage. Fortunately, pre-dialysis of the serum at pH 5.5 was found to enhance the ion exchange mechanism by removing the buffering effect of serum and allowing the slightly higher and unharmful pH to be used. The low molarity of this dialysate must also contribute to the maximal displacement of the counter-ions by the positively charged globulins, and the results of this single batch ion exchange showed that the globulins were extremely successfully removed by the procedure. These globulins could be recovered by elution with 0.14 M NaCl solution and the gelatin regenerated by displacement of the gradient ions and equilibration to starting conditions. The globulin eluted from the gel could constitute a source of human anti-HBc.

The dilute ion exchanged supernatant fluid contains the acidic serum proteins and HBAg. Separation of these two components is not necessary prior to detergent treatment of the Dane particles to release antibody-free cores. However, the action of the detergent is more predictable and rapid if the HBAg is precipitated from the serum proteins. Separation and concentration is relatively easily achieved with PEG precipitation. It was found that the pH should be raised to 8.6 to increase separation of HBAg from albumin as at the lower pH of 5.5, complete displacement of HBAg was not obtained.

Hepatitis B antigen without contaminating globulins,

and suitable for detergent treatment to release cores was produced by three steps; dialysis, ion exchange and PEG precipitation. Large amounts of donor serum were processed and treated with detergent. Isolation of the released cores by affinity chromatography of this detergent treated material on cyanogen bromide-activated-agarose beads (CN-Sepharose) with anti-HBc globulin as the ligand was attempted but failed to release detectable cores after elution with 1M NaCl or 4 M sodium thiocyanate. This may have been due to the destructive effect of the chaotropic ion on uncoated cores, or to non-specific absorption of released cores to the gel aggravated by inadequate numbers of cores.

Two reports in the literature indicate successful isolation of small quantities of Dane core particles from serum. Lipman et al., (1973)used gradient centrifugation with both a detergent (Nonidet-P40) layer above a Ficoll/sucrose D_2O gradient and a CsCl gradient. Although debated in the report, clear evidence of anti-HBc is present on the cores in micrographs of preparations which banded at 1.28 (CsCl) and these antibody-coated cores were non-reactive to anti-HBc. Some antibody-free cores were obtained at a density of 1.29 - 1.30 in caesium chloride and these reacted with concentrated anti-HBc in gel diffusion. More recently Fauvel et al., (1975) isolated HBAg by centrifugation of serum at 100 000 x g for 25 h over a 45% sucrose cushion followed by twice banding in CsCl. Dane rich fractions were identified and

further purified by sucrose density gradient, detergent (Tween 80) treated and the released cores separated by rate zonal centrifugation in a sucrose gradient. This material produced antibody-free cores which were used for immune electron microscopy.

The successful preparation of a large amount of core antigen from 5 l of plasma has been reported by Tsuda et al., (1975). These workers used a zonal rotor (Beckman CF 32) with a continuous flow rate of 1.2 l/h and a peripheral cushion of 60% sucrose to perform the initial isolation of Dane rich fractions. The fractions (300 ml) were further separated by large scale gradients of CsCl and sucrose in special rotors, treated with pronase, detergent (NP 40) and mercaptoethanol before complete separation of HBcAg from HBsAg by sucrose gradient was achieved. This HBcAg was used in an immune adherence haemagglutination assay to screen 215 sera for anti-HBc.

Despite the procedures developed by Lipman et al., 1973; Fauvel et al., 1975 and those in the present study the need for large amounts of pure core antigen for assay of anti-HBc may be obviated by the development of a micro-SPRIA assay by Purcell et al., (1974). Human anti-HBc, labelled with ^{125}I is used to detect the amount of added HBcAg bound by the test serum. The small amount of HBcAg needed is simply prepared by detergent treatment of washed Dane particle containing antigen. Because of the

specificity of the radio labelled anti-HBc, there is no need to remove HBsAg from the preparation.

The Dane particles and core antigen produced by the three methods of ultracentrifugation, gel filtration and ion exchange were found to be suitable for immune electron microscopic assay of anti-HBc and for studies of the antigenic complexity of the Dane particle.

Chapter 4

PREPARATIVE IMMUNO-ABSORBENT ELECTROPHORESIS

INTRODUCTION

Mead (1958) described a method for separating one from a number of soluble antigens by driving the mixture by electrophoresis through a gel column containing a gradient of antibodies to all the components except the one it was required to harvest.

This method has been applied to the separation of HBAg from human serum (Moodie and Polson, 1973). Anti-serum raised in rabbits to normal human serum which contained no HBAg detectable by counter electrophoresis or by electron microscopy was used. This rabbit anti-human serum was fractionated with PEG and the globulin fraction further purified by preparative electro-phoresis (Step 1) to remove components that migrate towards the anode. The buffer chosen was of low molarity and of pH 8.8 thereby balancing the negative charge of the globulins against the endosmotic flow. Agarose was used in the column in preference to agar as the elimination of the charged agaropectins in agar reduced the endosmotic flow (Polson and Russell, 1967).

The shape of the apparatus was modified from the U-shaped tube used by Mead (1958) to two communicating

vertical limbs (the "H-tube") which facilitated the pouring of agarose and the layering of material to be subjected to electrophoresis. Since the electrophoretic inhomogeneity of the immunoglobulins ensured the formation of a gradient in the gel, the prior establishment of an antibody gradient (Mead, 1958), was found to be unnecessary.

The technique is described in some detail, not only as an alternative method for preparing HBsAg from whole plasma but, since the separation is serologically dependent, as an immunoabsorbent filter on a preparative scale. All human serum components are immobilised in the gel by the rabbit antiserum so any components migrating through the gradient of antibodies in the gel may be considered to be abnormal.

The success and efficiency of this preparative technique required the assumption that normal serum components were not present on the surface of the HBsAg. Two opposing views had been expressed on this point.

Antigenic determinants related to normal serum proteins (albumin, pre-albumin, apolipoproteins and immunoglobulins) which were reported by Neurath et al., (1974) to be present on the HBsAg, were detected by affinity chromatography. Antisera to serum proteins were immobilised by cyanogen bromide linkage to Sepharose 4B. In their experiments HBsAg was absorbed to, and could be eluted from, the gel when the ligand was specific anti-

body to these normal serum proteins. These findings were challenged by Goudeau et al., (1974) who showed that absorption of HBsAg could be prevented by pretreating the immunoabsorbent with 1% bovine serum albumin and by using TRIS buffered saline of relatively high (0.5) molarity. Neurath's reply, indicated (that he felt) that contaminating serum proteins were present in the "partially purified HBsAg" used by Goudeau et al., and that these inhibited binding of HBsAg to the anti-normal human serum immobilised on the gel (Neurath, 1975). In considering the two conflicting reports, it is of interest and indeed of utmost importance to note that the antigen used by Neurath et al., (1974) in their original experiments was purified by a method that included glycerol gradient separation (Neurath et al., 1973 a and b). (Elaboration of the significance of this technical point will appear later in this thesis).

The use of the "H-tube" apparatus and the immunoabsorbent electrophoresis technique made it possible to contribute positively to the controversy because

(i) non-specific absorption was avoided by driving the antigen by electrophoresis through semi-solid agarose;

(ii) the HBsAg was unaltered and undamaged by prior fractionation procedures and

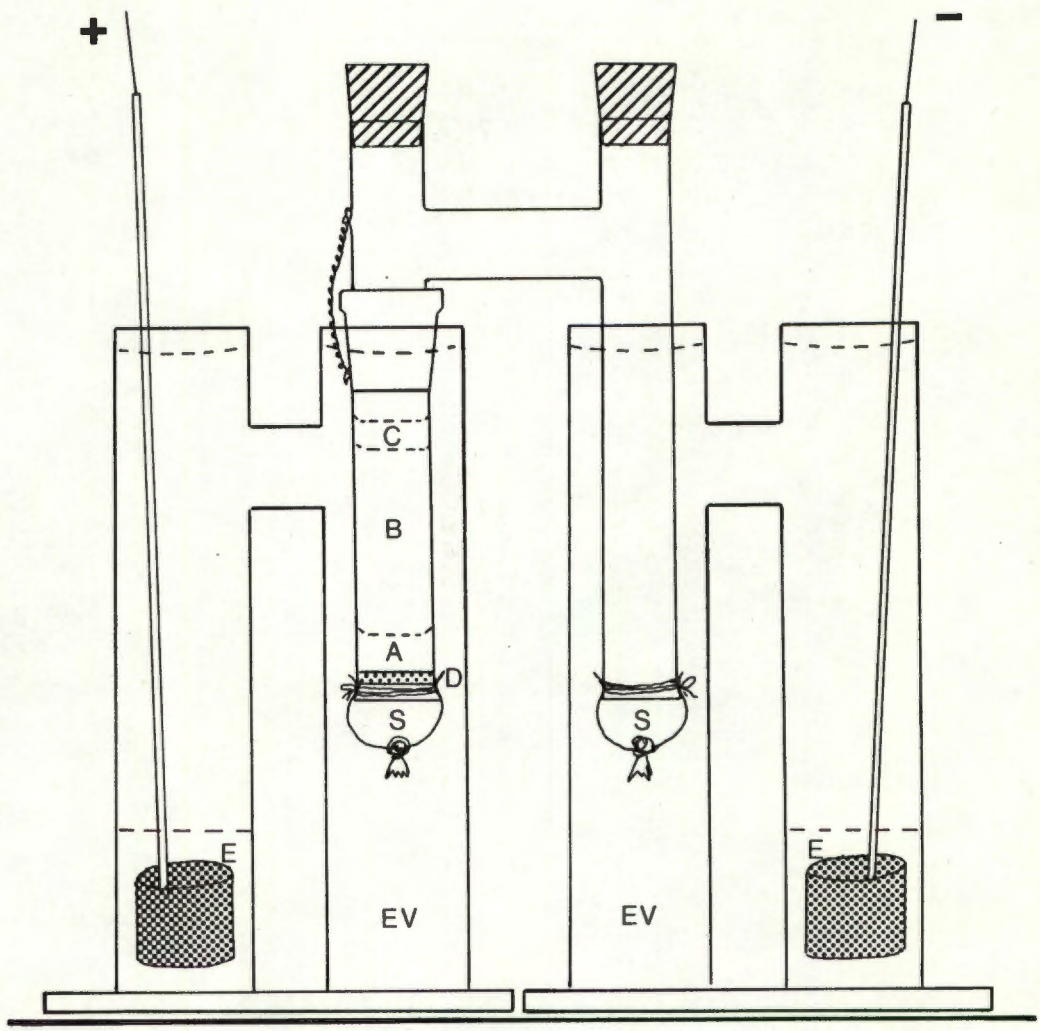
(iii) a gradient of antibodies to normal human serum components was present, so that saturation of antibody binding sites was unlikely. This should have ensured

that HbsAg was exposed to graded concentrations of rabbit anti-normal serum proteins.

METHODS

The "H-tube" apparatus used in this procedure is illustrated in Fig. 4.1. Two 20 cm x 2 cm vertical limbs were joined by a 7.0 cm horizontal tube of similar diameter. The left-hand limb was fitted with a tapered ground glass joint and bridged at its lower end by a No. 5 sintered glass filter disc (D). The lower openings of both limbs were flared to facilitate the attachment of cellophane dialysis tubing of appropriate diameter. The tubing was secured above with cotton thread and closed below by knotting the distal end. A small amount of buffer was introduced into the left-hand limb and forced by positive pressure through the sintered glass filter into the sac (S) to ensure that all the air in the sac was similarly sealed with cellophane and filled with buffer.

The "H-tube" was clamped vertically to retort stands and the lower ends immersed in separate vessels containing the buffer. Each vessel in turn communicated with an electrode vessel (EV) in which the silver/silver chloride electrode (E) ~~were~~ was placed and covered with a saturated solution of NaCl.



A	ANTIBODY GEL
B	SPACER GEL
C	ANTIGEN GEL
D	SINTERED DISC
E	ELECTRODE
EV	ELECTRODE VESSEL
S	CELLOPHANE SAC

FIG.4.1. IMMUNO-ABSORBENT ELECTROPHORESIS

Step 1

Agarose 2% was melted in 0.02 M Tris/glycine/ barbitone buffer, pH 8.8, and allowed to cool in a bath of water at 43°C. Anti-human rabbit globulin 4.0 ml was warmed to 43°C in the water bath and immediately mixed with an equal volume of the 2% agarose in buffer by inversion. This mixture of 1% solution of agarose, and 1/2 dilution of antibody in 0.01 M buffer was poured into the left-hand limb of the "H-tube" and allowed to gel (A). A spacer gel (B) of 8.0 ml of 1% agarose was poured on to the antibody-containing-gel and the column filled with buffer.

A direct current of 4 volts per cm was applied with the anode at the left-hand limb. Electrophoresis was maintained for 24 h. At the end of this period the current was switched off and the dialysis bag below the gel removed and the contents collected.

Fresh tubing was applied to the same limb, secured with cotton thread, and the tubing was filled with buffer and knotted under water to avoid air bubbles.

Step 2

The sample of HBsAg containing serum to be subjected to electrophoresis was warmed to 43°C and immediately mixed with an equal volume of molten 2% agarose at the same temperature. After removing the buffer from the

column, the mixture was poured on to the surface (C) of the spacer gel of the left-hand limb. When this had gelled the apparatus was filled with buffer and electrophoresis was recommenced at the same voltage as in Step 1 for a period of 48 h. At the end of this time, the contents of the dialysis sac of the anodal limb were carefully collected, and the walls of the dialysis membrane washed with a small quantity of buffer to ensure the recovery of any material that may have become attached to the wall of the cellophane sac.

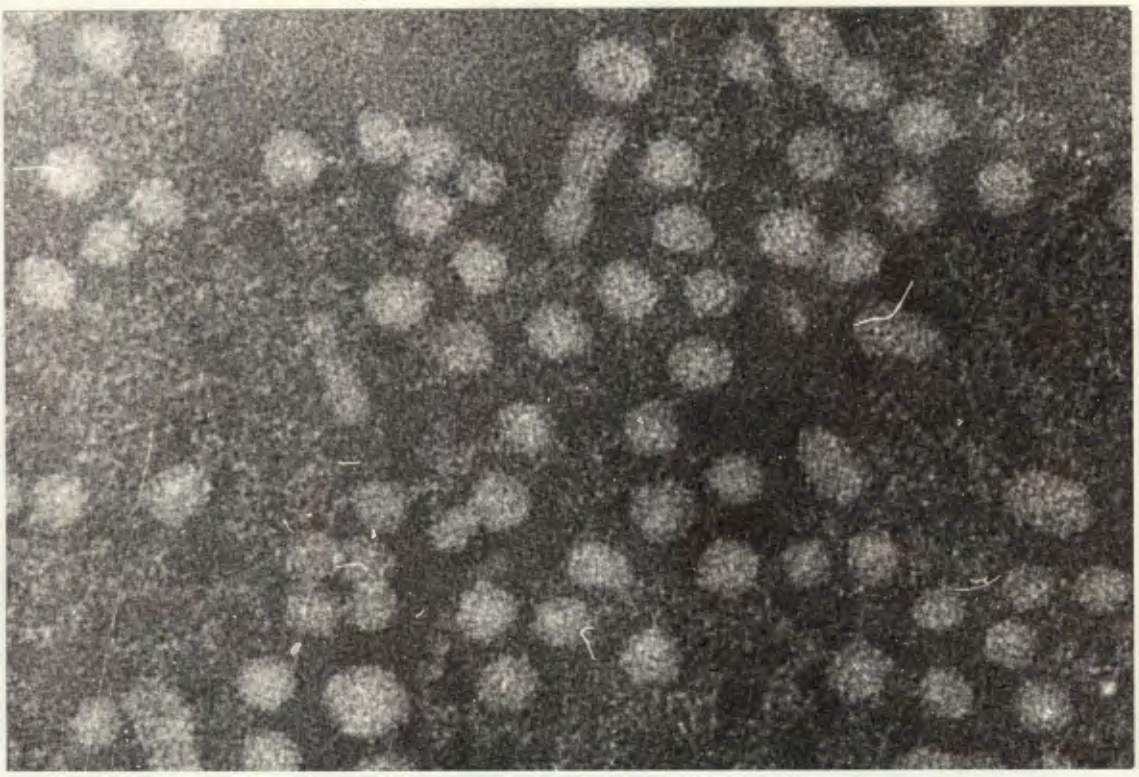
RESULTS

Step 1

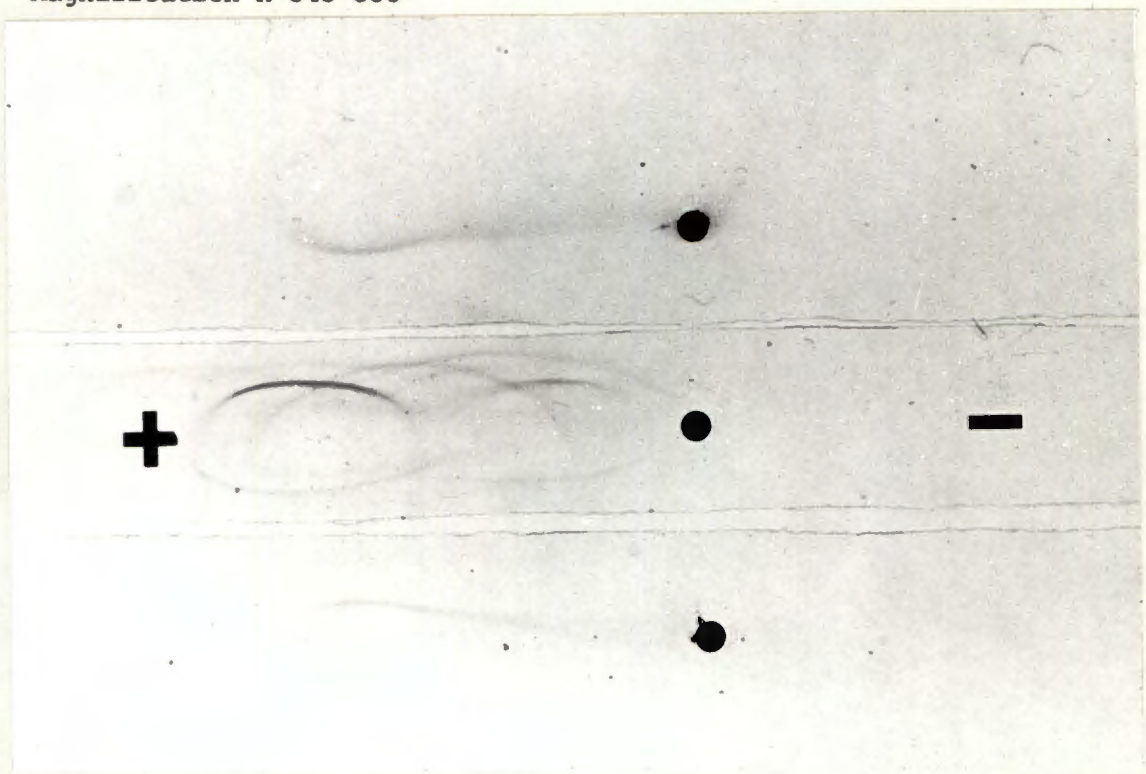
Analysis of the fluid collected from the dialysis sac at the end of Step 1 i.e. prior to the addition of the antigen containing material, invariably revealed only the faster migrating gamma globulin, small amounts of alpha and beta globulins and a trace of albumin.

Step 2

After completion of the preparative electrophoresis, fluid from the sac contained HB antigen on electron microscopic examination (Plate 4.1.a.) and this antigen corresponded in morphology with that of the starting material. IgG, IgM or IgA human immunoglobulins were not detected in this fluid by radial immunodiffusion



4.1.a. HBAg prepared by immuno-absorbent electrophoresis. Magnification x 340 000



4.1.b. Central portion of an immuno-electrophoresis plate. Outer wells contained fluid from dialysis sac of "H-tube" on completion of Step a. Central well: normal human serum. Troughs: horse anti-normal human serum.

PLATE 4.1.

in Tri-Partigen plates. However electrophoresis of the material from the dialysis sac did demonstrate a component extending from the point of application to the position of the β-1 lipoprotein, which on further analysis by immuno-electrophoresis using horse anti-human serum, proved to be a single component of wide electrophoretic heterogeneity (Plate 4.1.b.) and of uncertain nature but possibly derived from the HB antigen particles or perhaps soluble elements of the HB surface antigen.

DISCUSSION

The results obtained from a series of experiments, as exemplified by the one recorded above, indicated that the simplest method of preventing contamination of the HBs antigen by fast migrating components of the antiserum, was not to rely on purification of antiserum before mixing with the gel, but to use a preliminary electrophoresis to clear the antibody containing gel of anode migrating components before applying the mixture of antigens. This ensured that variation in buffer, pH and agarose purity were rendered harmless since any components of the rabbit antihuman serum entering the sac were removed before the electrophoresis of the HB antigen containing material commenced.

The presence of albumin and faster migrating gamma globulins in the sac at the end of the preliminary electrophoresis confirms the necessity of the step. During

this phase, the slowly migrating globulins were moved upwards by endosmosis into the spacer gel from the underlying antibody containing gel, and due to the inhomogeneity of the antibody, a gradient of antibody was formed. The second step of the technique was performed with HBAg positive serum incorporated into a gel layered over the antibody gradient and driven into the gradient by electrophoresis. Bullet-shaped precipitin bands which developed in the spacer gel during electrophoresis were visible evidence of the reactions between normal human serum components and the immunoglobulins in the rabbit antiserum. Optimal proportions were estimated by preliminary one-dimensional Laurell electrophoresis with trial dilutions of the anti-human serum in the gel. From the height of the resultant precipitin lines, it was possible to estimate the correct dilution of antiserum to be used in the "H-tube" column. This was not felt to be a critical determination as the length of the gel in the limb of the column is sufficient to accomodate a wide variety of reactions at differing optimal proportions.

Partially purified and concentrated HBAg preparations would no doubt have yielded more HBAg without increasing the antigenic load on the antiserum in the gel, but fractionation procedures were specifically omitted in order to avoid the risk of exposing serum components normally masked beneath the surface structure of HB antigen. Should such determinant sites exist and be revealed by the manipulations involved in fractionation,

the HB antigen would become immobilized in one or other of the precipitin reactions in the antiserum-containing gel.

This procedure of immunoabsorbent electrophoresis may therefore be regarded as one of immunological filtration. It does not depend on the availability of an antiserum with mono-specificity to the desired component but rather on an effective polyvalent serum to the spectrum of unwanted components in the mixture.

This technique has a wide application for isolating and concentrating abnormal components from serologically reacting normal animal or plant fluids. It has been used for separation of tobacco mosaic virus (TMV) from wild cucumber virus by incorporating one or other of the homologous antisera into the gel. Not only was separation achieved but the procedure resulted in the concentration of TMV and the removal of plant protein from the suspension (Polson and Moodie, unpublished data).

In so far as the experiments with HB antigen in human serum were concerned the advantages of the method were apparent in that with a single antiserum it was possible to isolate and purify the HB antigen from a mixture of serum proteins which were held back by their respective antibodies and entrapped as antigen-antibody precipitates in the gel.

The isolation of HBAg from this system gave an important indication that normal human serum components are not present on the surface of undamaged HB antigen.

Chapter 5

AFFINITY CHROMATOGRAPHY

INTRODUCTION

Affinity chromatography may be used to isolate either component of a reversibly reacting system. One component is coupled to an insoluble matrix and must at the same time retain its specific binding capacity.

Concanavalin A (a plant lectin) by its affinity for certain glycoproteins present in HBsAg (Burrell et al., 1973) will combine reversibly with certain surface components of the antigen (Cawley, 1972; Neurath et al., 1973c). This affinity is not sufficiently specific for the purification of HBAg from plasma, since approximately 15% of serum proteins, notably alpha globulins, ceruloplasmin, pre-albumin, IgM and IgA immunoglobulins will also combine with Concanavalin (Leon, 1967).

A better system, dependent on the immunological specificity of HBAg, either surface or core antigen, requires the coupling of the appropriate antibody to an insoluble matrix with retention of activity. Provided that the antibody is specific, that steric hindrance does not occur and that dissociation of the bound substance from the ligand can be achieved without damage, then adequate yield and purity could be expected.

Sepharose 4B with its open pore size displays virtually all the desirable features of a matrix for the preparation of bed materials for affinity chromatography (Cautrecasas and Anfinsen, 1971). CNBr-activated Sepharose is available commercially, or beaded agarose may be activated with CNBr by the method of Porath et al., (1967) as modified by Grabow and Prozesky (1973). The nature of the reaction product is not known, but it is believed that cyanogen bromide reacts with the hydroxyl groups of the agarose to form imidocarbonates. These groups react during coupling with the amino-groups of the ligand to form covalent linkages, probably with multi-point attachment.

After reaction between the ligand and the antigen, the unbound material is washed away and chaotropic ions may be used to reverse the reaction. The covalent linkage binding the ligand to the matrix is not affected by this procedure and the antigen is eluted leaving the antibody attached to the bed.

METHODS

One gram of the freeze dried gel (CNBr-activated Sepharose 4B, Pharmacia, Fine Chemicals, Uppsala, Sweden) was washed for 15 min with 10^{-3} M HCl solution over a sintered glass filter. The swollen gel (3.5 ml) was poured into a small glass column 1.5 cm in diameter and 14 cm long, fitted at its lower end with a sintered

glass filter and closed with a stopcock. The gel was washed with 200 ml of coupling buffer (0.1 M carbonate/ bicarbonate pH 9.4).

The procedure required two steps, (i) the coupling of the 'antibody' to the matrix and (ii) passing the 'antigen' through the column. The 'antibody' consisted of an antiserum from a baboon which had been immunized with purified HBs antigen. The 'antigen' was the serum from a chronic carrier known to have all three morphological forms of the HB antigen present.
Step (i). Two ml of unfractionated baboon antiserum to purified HBAg was added to the gel in the column, the stopcock was closed and the mixture gently agitated in a shaker at room temperature for 2 h. After coupling, the column was washed with 0.1 M $NaHCO_3$ buffer solution containing 0.5 M NaCl (200 ml) and reacted with a slow flow (40 ml/h) of 1 M ethanolamine pH 8.0 for 2 h. Finally the material was washed with 3 cycles of 200 ml 0.1 M Na acetate buffer containing 1 M NaCl pH 4.0 followed by 0.1 M borate buffer containing 1 M NaCl pH 8.0 and completed with a wash of PBS pH 7.2.
Step (ii). HB antigen-positive serum of a chronic carrier was diluted with PBS 1:10 and 100 ml of this diluted serum was allowed to flow through the column at a rate of 40 ml/h. The column was then washed with 100 ml PBS pH 7.2 at the same flow rate.

Elution was performed with (a) 20 ml of freshly

prepared 4 M NaI at a flow rate of 10ml/h; (b) 4M NaSCN under the same conditions of flow. The eluates from the two solutions were kept separately and dialysed immediately against running distilled water for 4 h. The 20 ml of dialysate was freeze dried and re-suspended in 1.0 ml of PBS pH 7.2.

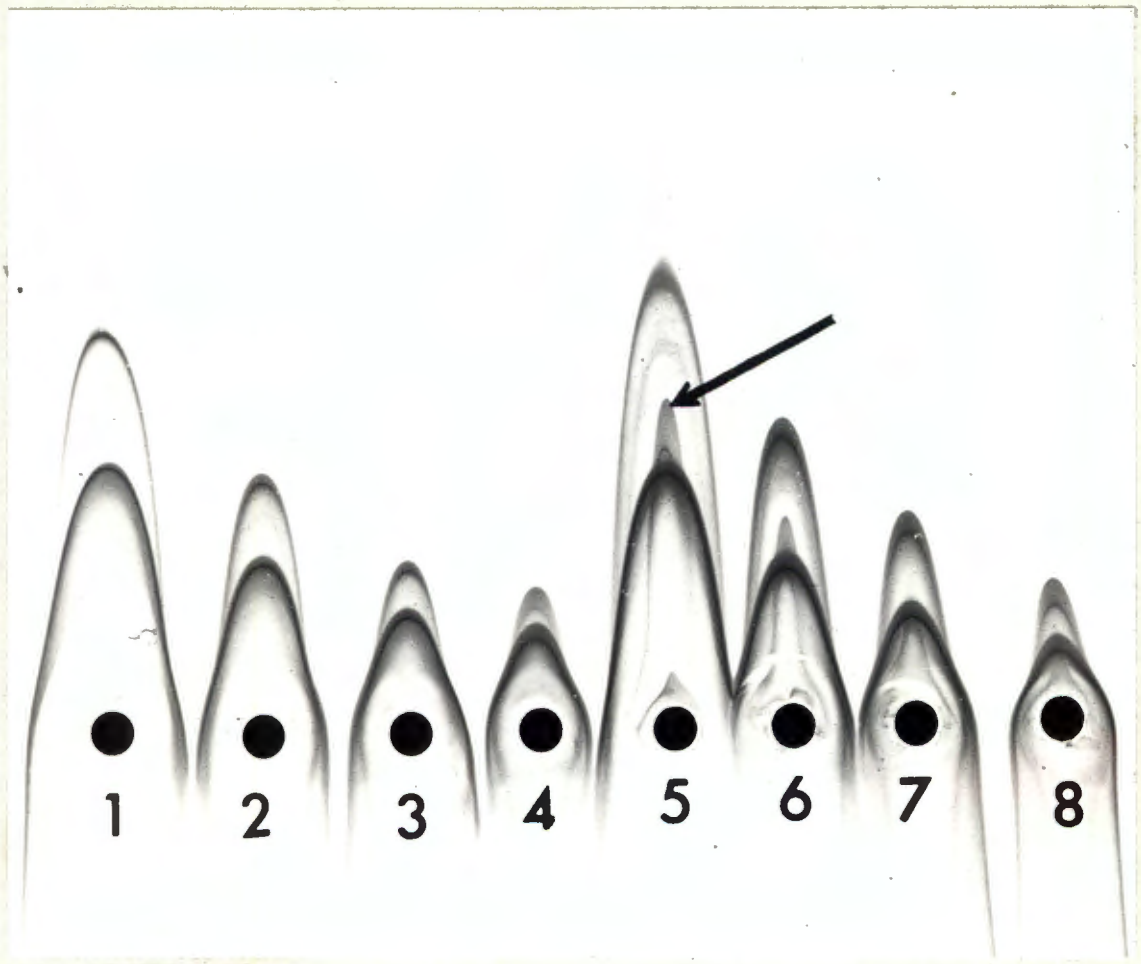
The pooled eluates from two consecutive experiments were used to immunize a sheep by multiple subcutaneous injections on two occasions. The sheep was bled three weeks later and the separated serum stored at 4°C.

Globulin fractions prepared from the serum of the immunized sheep were diluted 1:10 for use in the gel of one- or two-dimensional Laurell electrophoresis plates for evaluation of anti-HBAg activity and for the detection of any contaminating antibody.

RESULTS

Four techniques were used to assess what had been achieved by the affinity chromatography: - electron microscopy, electrophoresis, immuno-electrophoresis and animal inoculation.

The diluted sample of HBAg-positive donor serum after passage through the chromatography column was found, by electron microscopy, to contain abundant



5.1.a. One dimensional Laurell electrophoresis.

1% agarose gel contains 1:10 dilution of globulin prepared from hyperimmune sheep serum by precipitation with PEG. Sheep was immunised with HBAg prepared by affinity chromatography using baboon anti-HBs serum.

Wells 1 - 4 contained $\log^2$ dilutions of HBAg negative whole serum.

Wells 5 - 8 contained similar dilutions of HBAg +ve material. Arrow indicates HBsAg precipitin arc.

PLATE 5.1.

antigen with a similar distribution of the three morphological forms of the antigen as that seen in the original undiluted serum. There was no apparent greater reduction in antigen concentration than could be accounted for by the dilution. Examination of the eluate after dissociation with sodium iodide revealed the presence of small spheres only. Material from the subsequent dissociation with sodium thio-cyanate showed in addition to the small spheres, some filamentous forms and occasional Dane particles. The total yield of HBAg in the eluates was considerably less than that present in diluted serum introduced into the column.

Electrophoresis in agar gel and immuno-electrophoresis with horse anti-human serum however, failed to detect the presence of any contaminating proteins in the freeze dried concentrates eluted from the chromatography column.

This apparent complete separation of HBAg components from human serum components could not be sustained when the serum from the sheep immunized with the eluates was examined. The globulin fraction from this antiserum was evaluated in a series of Laurell,(1965) electrophoresis plates and gave the following results:

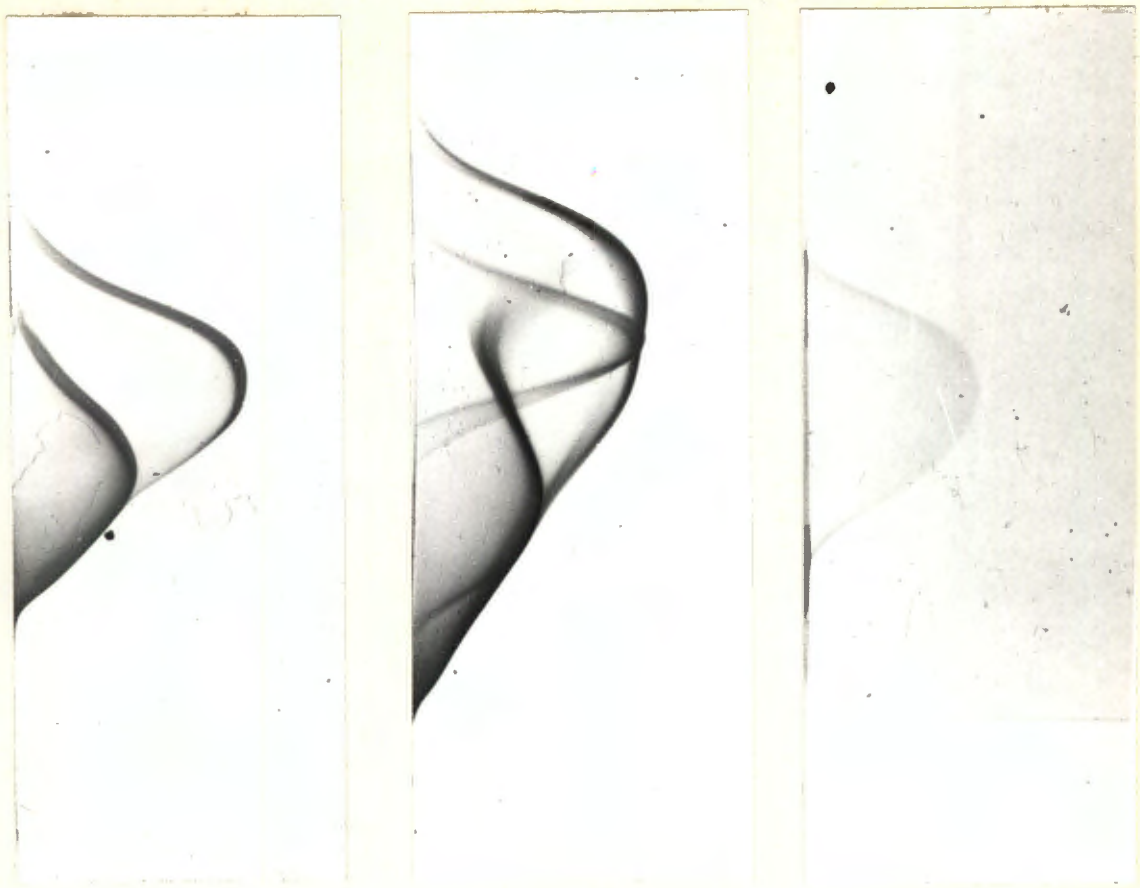
When normal human serum in two-fold dilutions was migrated by electrophoresis into a gel containing a 1:10 dilution of the immune sheep globulin, a series of

characteristic bullet shaped lines of precipitation appeared (Plate 5.1.a., wells 1 - 4), but when the HBAg-positive serum was treated in the same way (Plate 5.1.a., wells 5 - 8) an additional precipitin arc developed indicating anti-HBs activity. These antigen-antibody reactions were further resolved by two-dimensional Laurell electrophoresis (Plates 5.2.a. and b.) wherein the HBsAg was clearly responsible for the extra precipitin line while the other precipitin lines present in both sera corresponded with gammaglobulin and one or two components in the region of the alpha and beta globulins. The immune globulin was absorbed with glutaraldehyde insolubilised normal human serum proteins by the method of Ternynck and Avrameas, (1976), to produce a monovalent HBsAg specific antiserum (Plate 5. 2.c.)

DISCUSSION

This method of isolating HBAg from whole serum relies on the specificity of the antibody. It is theoretically possible to isolate selectively any portion of the antigen that has a different and specific immuno-reactivity.

In the light of the successful isolation of the HBAg from all but trace amounts of two or three serum components in the experiments recorded above, several attempts were made to isolate the cores from Dane



5.2.a. 5.2.b. 5.2.c.

Two dimensional Laurell electrophoresis. Gel of the second dimension contains 1:10 dilution of hyperimmune sheep globulin (5.2.a. and 5.2.b.). The gel in 5.2.c. contains the identical globulin after absorption with insolubilised normal human proteins.

5.2.a. HBAg -ve serum (first dimension). Precipitin lines indicate anti-normal human serum activity.

5.2.b. HBsAg +ve serum (first dimension). Additional precipitin line indicates anti-HBs activity.

5.2.c. HBsAg +ve serum (first dimension). Single precipitin line indicates sheep globulin monospecific after absorption.

PLATE 5.2.

particle-rich human serum. For these experiments the anti-HBc immunoglobulin was purified by PEG precipitation from the serum of a chronic carrier whose serum had been shown by electron microscopy to be core-reactive by the formation of core complexes. This antibody was coupled to the insoluble matrix. Dane particle cores, free of antibody, were prepared by detergent treatment of Dane particle-rich serum and concentrated by PEG precipitation as described in Chapter 3. Eluates from chromatography columns flooded with this core antigen were unproductive of anything but occasional cores. Some of the reasons for the failure of these experiments are discussed below.

In practice it was important to ensure that high ionic strength in the buffer solution should be maintained during coupling and washing in order to reduce protein to protein adsorption in conditions of low salt concentration. This was achieved by adding 0.5 M NaCl to the buffer solutions in the coupling and washing cycles. After coupling had been completed, residual active groups on the gel could be blocked by adding an excess of a small primary amine such as glycine or ethanolamine, and in these experiments 1 M ethanolamine was used. After the remaining active groups have been blocked, thorough washing with alternatively high and low pH buffers is recommended, in addition the molarity was kept high and 1 M NaCl added to the acetate and borate buffers. This was necessary for the thorough removal of traces of non-

covalently adsorbed materials. These adsorbed materials were not detected by electrophoretic analysis of the eluates.

The nature of the antigen used in these experiments placed restrictions on the technique for the best results in affinity chromatography. The antigen could not be prepared by any method making use of low pH conditions or any digestive process before elution. The more usual method of washing the attached antigen with three cycles of low and high pH buffers had to be omitted for this reason. Many workers (Kim and Tilles, 1973; Nath et al., 1976 and others) have shown that to prepare a monovalent serum it is necessary to dissociate attached serum proteins from the surface of the antigen.

The fact that the eluates injected into the sheep gave rise to antibodies to three serum components in addition to the HBs antigen after affinity chromatography may be explained in one of several ways. The contamination of the antigen in the eluates by these serum components may arise from the donor serum, in which case the purification was not complete. The serum components may arise from the baboon serum used as the ligand which may have been partially eluted from the bed material by the strong dissociation conditions under 4M NaSCN. It was shown that the baboon serum reacted in the same manner on immuno-electrophoresis as human serum when tested against horse anti-human serum. It was thus not possible to

differentiate the serum proteins of the two primates by this test.

The third possibility is that the normal serum components to which antibodies were formed in the sheep, may have been adsorbed to the surface of the HBsAg or are perhaps an integral part of it. The latter concept is discussed at greater length in Chapter 8.

Chapter 6

DENSITY GRADIENT ZONE ELECTROPHORESIS

INTRODUCTION

Zone electrophoresis utilises the movement of a charged particle in an electric field. The direction and rate of movement in a given system is dependent on the net charge on the particle and is proportional to the charge density. Proteins, depending on the pH of the system, have either a net positive or negative charge. At their iso-electric point these charges are equal, resulting in a zero migration. Proteins of similar charge will move in a fluid medium in similar zones, regardless of size differences and proportional to their charge densities. This property has been used to separate mixtures of proteins, and mixtures of viruses and proteins (reviewed by Polson and Russell, 1967).

Svensson and Valmet, (1955) introduced a zone electrophoresis apparatus adapted for use with a density gradient in solid supporting media. This was modified by Cramer et al., (1957) by using a sucrose density gradient, in the study of mouse encephalitis virus. Changes in density due to temperature gradients were rendered innocuous by the presence of the density gradient and convection currents were minimized. Polson and Cramer, (1958) further simplified this

apparatus. Phenol red was introduced as a marker with the sample under investigation because of its high electrophoretic mobility. Van Regenmortel, (1960) used the symbol R_ϕ as an index of relative electrophoretic mobility expressing the ratio between the distance travelled by the test material and the phenol red. The ratio R_ϕ is valid under standard conditions and is therefore independent of temperature, concentration of the test material or column width.

The apparatus described by Polson and Cramer, (1958) and Polson and Russell, (1967) was used with minor modification to study HB antigen containing serum to confirm (or otherwise) the degree of electrophoretic inhomogeneity of HBsAg reported by Howard and Zuckerman, (1973) and to compare the migration index of the antigen with that of normal human serum proteins. Millman et al., (1970) had demonstrated the formation of a precipitin arc by immuno-electrophoresis when human anti-HBs was placed in the trough. The position of the antigen was shown in relation to the pattern of precipitins developed in a similar gel when whole serum was migrated by electrophoresis and horse anti-human serum allowed to diffuse from the trough.

Density gradient zone electrophoresis provided a method of expanding the electrophoretic pattern of serum by the analysis of 20 or more fractions and at the same time under standard conditions examining the electro-

phoretic mobility of (i) HBsAg +ve whole serum (ii) partially purified HBsAg prepared from whole serum by differential ultracentrifugation, and (iii) Mucasol treated partially purified HBsAg.

METHODS

Antigen

HBsAg was demonstrated in the serum by electron microscopy. The samples of partially purified HBsAg were obtained by sedimenting 2.0 ml of whole serum at 60 000 x g for 90 min. The pellet was resuspended in 2.0 ml of the buffer or in 2.0 ml of 2% Mucasol in the buffer. These samples were held at 4 °C for 5 days before use.

Apparatus

The apparatus is depicted in Fig. 6.1. and consists of an upright column (B) diameter 2.5 cm, with a vertical side limb (C) and stopcock (F). The upper ends of the columns were connected by a right angled tube to the buffer reservoirs (D) and (E). The lower end of the column (B) was provided with a side vent (A) and was sealed into a length of thick glass capillary tubing (G). The capillary tubing was connected to a length of rubber tubing of appropriate diameter equipped with clamp (H). Finally this rubber tubing was connected to the lower exit from a pair of mixing Ehrlenmeyer flasks connected

in series. The flow between the flasks was controlled by capillary tubing (J) and clamp (K).

Procedure

1. Veronal buffer 0.05 M, pH 8.6 was slowly added to the apparatus using the upper end of the column (B). It was important that no bubbles were formed while the pouring of the buffer was in process. To achieve this and to ensure that no air bubbles were left in contact with the glass walls of the apparatus, the tubes were cleaned meticulously beforehand, using a detergent, and removing all traces of this detergent to avoid its effect on the antigen.
2. A 40% solution of sucrose in veronal buffer was allowed to flow into the apparatus through side vent (A) until the level in the vertical limb was at X.
3. Buffer was added to the column (B) from above until the sucrose solution had been displaced downwards to about 2.0 cm above the capillary tube. This displaced the sucrose solution upwards about as far as the stopcock of the vertical limb on the right-hand side.
4. The stopcock was closed.
5. A little of the 40% sucrose was run out of the column through the capillary tubing (G), in order to sharpen the top edge of the sucrose boundary and to bring it level with the top of the capillary tube.
6. The mixing flasks were now connected, the higher flask containing 170 ml of 40% sucrose and the lower 160 ml

of plain buffer and a magnetic stirrer. The lower flask was sealed with a rubber bung and the lead to the column was milked to remove any air bubbles.

7. The clamp (K) between the flasks was removed and the magnetic stirrer started.

8. The tap at (H) was opened to allow the sucrose gradient to flow very gently into the column. In this fashion a gradient of increasing density of sucrose, was formed, displacing the buffer upwards as it extended.

9. The gradient was run until about 20 ml of sucrose were left in the flask.

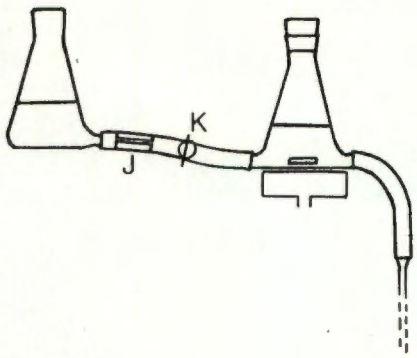
10. Approximately 5 ml of the lower part of the gradient was run off the column through the capillary (G) to ensure a concentration of about 35%.

11. The sample was then dissolved in 35% sucrose and the density adjusted to ensure that a drop of the sample sedimented in 35% sucrose and floated on 40% sucrose.

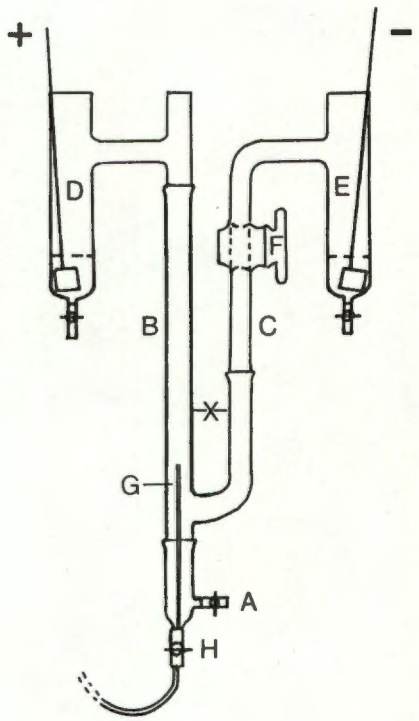
12. 2 ml of the sample, to which a pinch of phenol red was added, was slowly introduced through capillary (G). The sample had a density of approximately 37% sucrose and as the most dense lower part of the gradient was about 35%, the 40% sucrose formed a shelf on which the sample was based. These sharp density shelves prevented mixing.

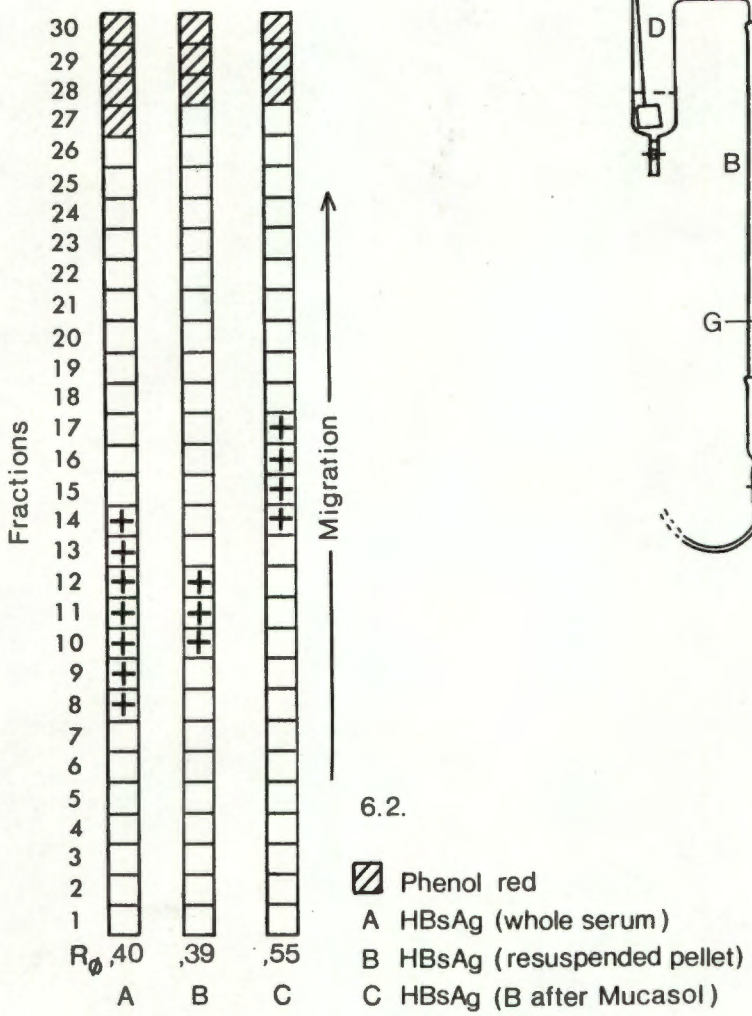
13. Reversible silver electrodes (Polson and Russell, 1967) were used in the electrode vessels and immersed in a band of saturated NaCl in the buffer.

14. The large stopcock in the apparatus was opened gently and the sample balanced by removal of buffer from the appropriate buffer chamber to lie about 5 cm above



6.1.





6.2.

Distribution of HBsAg in fractions after Zone Electrophoresis

FIGS. 6.1. and 6.2. ZONE ELECTROPHORESIS

the top of the capillary tube.

15. Cold air from a fan was directed onto the apparatus to minimize heating.

16. The apparatus was allowed to stand for two hours to allow diffusion to establish a continuous gradient through the sample layer.

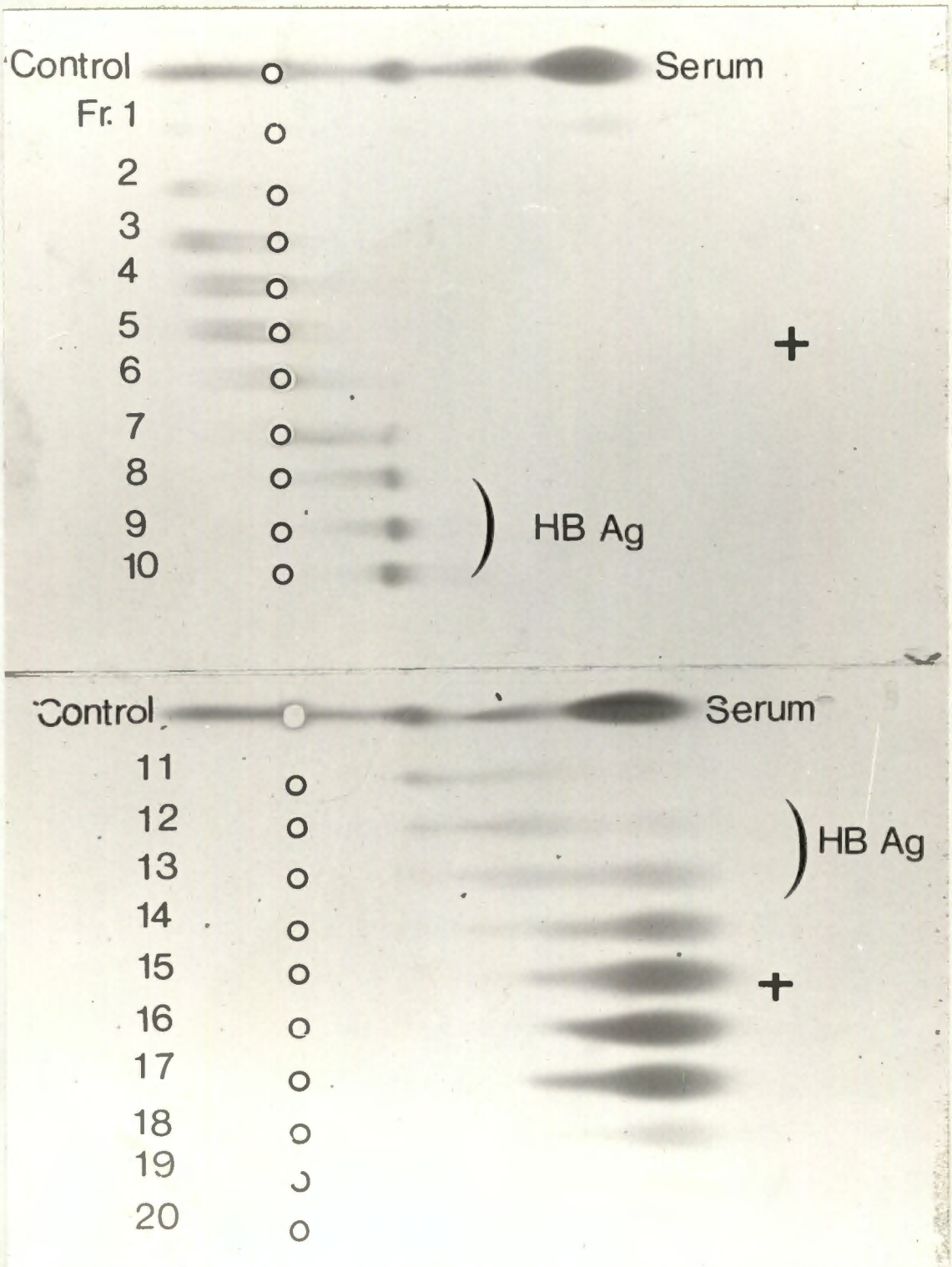
17. A voltage gradient of 3 - 4V/cm was applied which in these conditions resulted in a flow of 20 m amp which was maintained for 20 h.

18. At the completion of the experiment fractions were taken from the capillary tube at the base of the column, each sample measuring approximately 1 ml.

19. Each fraction was tested for HBsAg by counter current electrophoresis (CEP) in agarose gel with baboon anti-HBs in the anodic well. Those fractions which were positive for HBsAg by CEP were examined electron microscopically. Each fraction was subjected to electrophoresis in agarose gel.

RESULTS

The electrophoretograms of the fractions of HB antigen-containing whole serum, taken from the density gradient zone electrophoresis column are presented in Plate 6.1. A control normal serum was included with each batch of analyses for reference. In the earlier fractions the gamma globulins are seen to be clearly separated from the faster migrating components and from the albumin.



Electrophoretogram of fractions from HBAg-positive serum following zone electrophoresis.

PLATE 6.1.

Each fraction was examined also by counter electrophoresis for the presence of HBsAg. It will be seen that the antigen was detectable in fractions No. 8 through to No. 14 indicating a considerable measure of electrophoretic inhomogeneity and that it was associated with the alpha and beta globulins. Using the mid-point of the antigen-positive zone for comparison with the migration of phenol red the R_{ϕ} value was calculated to be, 0.40.

Two other preparations were examined under identical experimental conditions. HBAg prepared from the same serum (see above) by differential ultra-centrifugation, with the final sedimented pellet suspended either in i. buffer (Veronal 0.05 M, pH 8.6) or in ii. 2% Mucasol in the same buffer at pH 8.6.

Analysis of the fractions from the zone electro-phoresis column of these two preparations of antigen revealed important differences. Electrophoreses were less informative than before because of the marked reduction in serum proteins following centrifugation of the antigen. Counter electrophoresis of each fraction against anti-HBs (Fig. 6.2.) showed, as expected, that there was no difference in the electrophoretic mobility of the HB antigen and a similar R_{ϕ} value of 0.39 was obtained. The inhomogeneity was less since the antigen was confined to fractions Nos. 10, 11 and 12. The Mucasol treated antigen however had acquired an increased

electrophoretic mobility with a significantly higher R_ϕ value of, 0.55.

Lastly, the fractions giving positive results in the counter electrophoresis tests were examined by electron microscopy. The original whole serum was found to contain large numbers of all three morphological forms with occasional small complexes of Dane particles. No alteration in the distribution of the morphological forms of the HBAg was found in the electrophoretic fractions except that the Dane particle complexes were seen only in the slowest migrating fractions of the antigen-positive zone.

DISCUSSION

The results indicated that HBAg is inhomogeneous in migration in an electrical field in a sucrose density gradient. The heterogeneity under these conditions is dependent on charge density differences. The migration of antigen in this system, either in whole serum or as prepared from whole serum by ultracentrifugation was constant when expressed as a ratio of the migration of phenol red. Thus the presence of whole serum components did not influence the R_ϕ of the antigen. Gel electrophoresis of the antigen-positive fractions from the zone electrophoresis column showed that this apparatus did not separate HBAg from serum components even when considerable increase in the span of the apparatus was considered.

This is in contrast to the report of Howard and Zuckerman, (1973) who were able to purify small spherical antigen from partially fractionated HBsAg positive serum by electrofocusing in a pH gradient formed by a mixture of carrier ampholytes.

The alteration in R_ϕ for detergent treated antigen indicated that the charge density had been increased to give a greater net anionic charge. This alteration by the detergent may be due to the removal of lipoprotein or to other less negatively charged (slowly migrating) proteins from the outer layer of the HBsAg or to the reduction of the ratio of the amino to carboxyl groups of the exposed protein. It is shown elsewhere in this thesis that detergent treated antigen retains its immunological identity indicating that severe disruption of the exposed protein determinant groups was unlikely. On the other hand it was also shown (see later) that detergent treatment exteriorises immunoglobulins but this might be expected to slow the migration of the antigen. This is also suggested by the behaviour of naturally occurring complexed HBsAg untreated serum, as these complexes were only found amongst the slowest migrating HBsAg fractions. The presence of gamma globulin in the complexes reduces the net negative charge and so exposure of IgG was not responsible for the increased R_ϕ value obtained after detergent treatment.

Other workers (Burrell, 1975; Neurath et al., (1974)

have shown the presence of several normal serum proteins associated with the surface antigen after partial purification by methods which have been shown to be damaging (Stannard and Moodie, 1976). In the series produced by Neurath et al., (1974) the largest category of serum components found in association with the antigen was that of the apo-lipoprotein group of substances. These would be detergent labile and their removal would allow the higher charged proteins such as albumin (also shown to be present) to increase the net negative charge on the surface of the HBAg particle and so produce a faster migration with a correspondingly high R_ϕ value.

Chapter 7

IMMUNE ELECTRON MICROSCOPY

INTRODUCTION

The advantage of immune electron microscopy over conventional methods of detecting immunological reactions lies with the direct visualisation of the immune complex. It is generally thought that assays involving radioactive techniques have a higher sensitivity for detecting immune reactions, but immune electron microscopy, where the antigen is of sufficient size to allow easy detection, although perhaps lacking this extreme sensitivity has the unique advantage of absolute specificity. In most of the instances quoted in this work, the technique was used for the demonstration of antibody to "morphological" sub-groups (as opposed to serological subgroups) of the HB antigen and was at times the only method for obtaining information of this reaction; it frequently circumvented the necessity of full-scale purification of the subgroups.

Almeida et al., (1971) was the first to use this technique in the field of hepatitis B antigen analysis to demonstrate the separate specificity of the Dane core. It was only some years later that Hoofnagle et al., (1973) were able to confirm this observation by complement fixation. (see Chapter 3. Discussion).

Immune electron microscopy was used as a sophisticated technique for immunological reactions involving the antigens of hepatitis B virus associated components and their respective antibodies.

METHODS

Antigen i. HBsAg in human serum concentrated by ultra-centrifugation

ii. HBcAg produced from i. (above) and released by treatment with 1% Mucasol or 0.025% deoxycholate and iii. HBcAg extracted from liver cell nuclei of a patient with fulminating fatal hepatitis.

Antibodies

Immune serum from a baboon inoculated with purified HBsAg (Natal Institute of Immunology, 129 Princes Street, Durban) and human sera containing anti-HBs or anti-HBc or both. Sera from chronic carriers were also used as a source of anti-HB_{III}.

One "grid dose" of antigen comprising all morphological components was resuspended in 0.25 ml PBS after concentration and washing by ultra-centrifugation. An equal volume of the appropriate antibody preparation was added and the mixture dispersed by immediate inversion of the centrifuge tube. Incubation at 37°C for 1 h was followed by overnight storage at 4°C. A control sample utilising the same antigen but incubated with PBS instead

of antiserum was included. The mixtures were then centrifuged at 50 000 x g for 60 min to sediment the antigen, and washed with phosphate buffer by two further cycles of the same force and duration before negative staining for examination in the microscope.

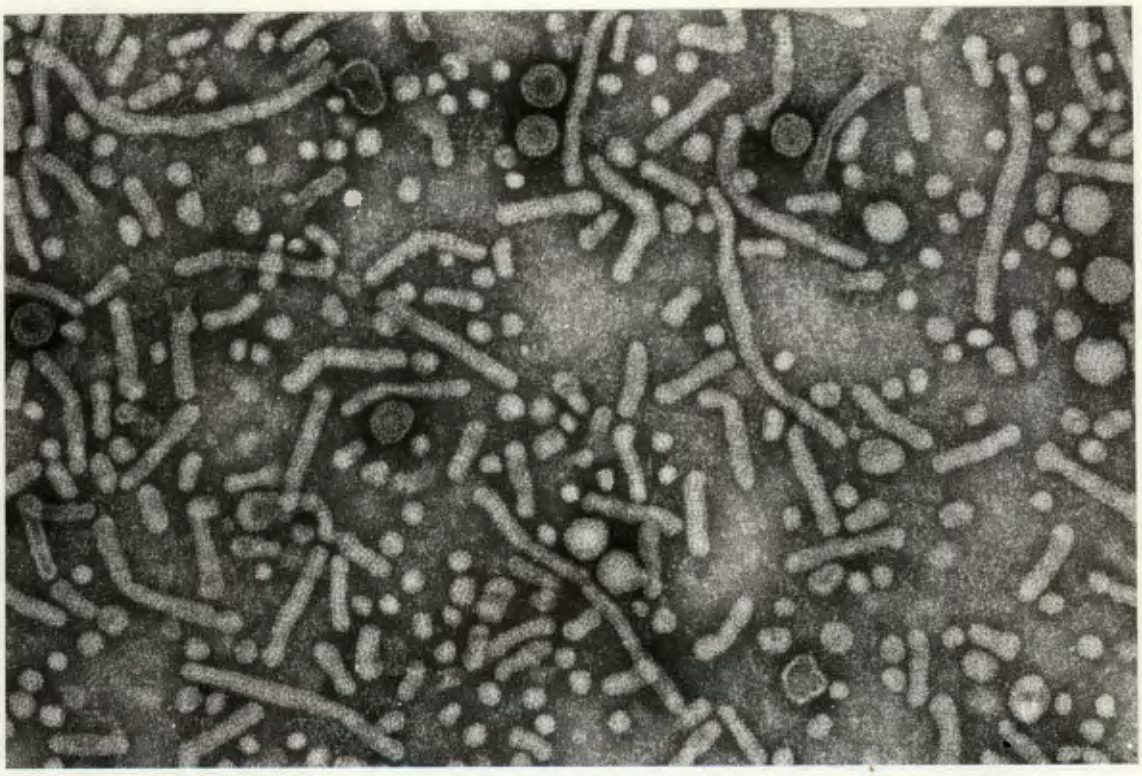
RESULTS

Antigen Control (HBsAg)

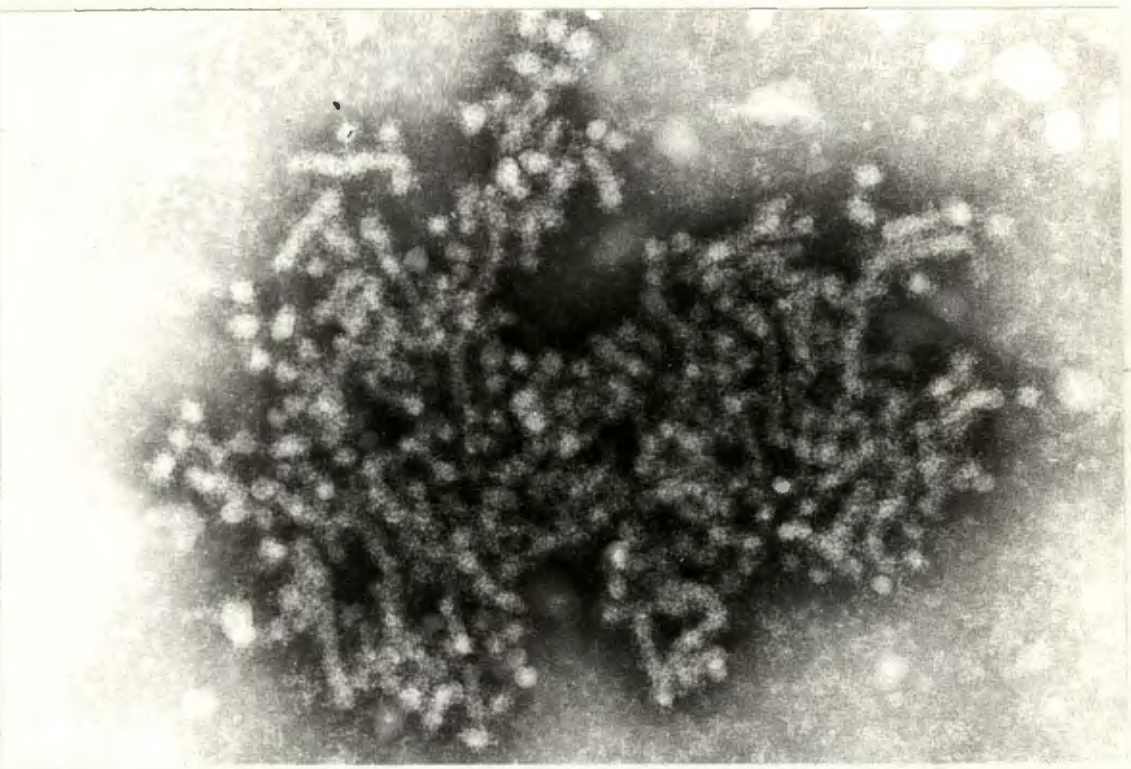
Hepatitis B antigen suspended in PBS (Plate 7.1.a.) showed the three components, spheres, tubules and Dane particles scattered at random and without attached immunoglobulin filaments.

Antibody to HB Surface Antigen (anti-HBs)

Appropriate dilutions of specific animal antisera from baboons or sheep, or of human sera containing antibody to the surface of hepatitis B antigen, when incubated with HBsAg produced immune complexes such as those represented in the micrograph (Plate 7.1.b.). Optimal concentrations of the reactants were demonstrated by the involvement of virtually all the particles of surface antigen present in the sample and by the cross-linking of the antigen by strands of immunoglobulin to form moderately sized discrete immune complexes. A preparation of antigen, but treated with 1% Mucasol in PBS (to strip the Dane cores of their coats) prior to



7.1.a. HBsAg control. Prepared from serum by differential ultracentrifugation and incubated in PBS. Magnification x 120 000



7.1.b. HBsAg aggregated into immune complexes by incubation with baboon anti-HBs. Fluffy appearance is due to excess of antibody. Magnification x 120 000

PLATE 7.1.

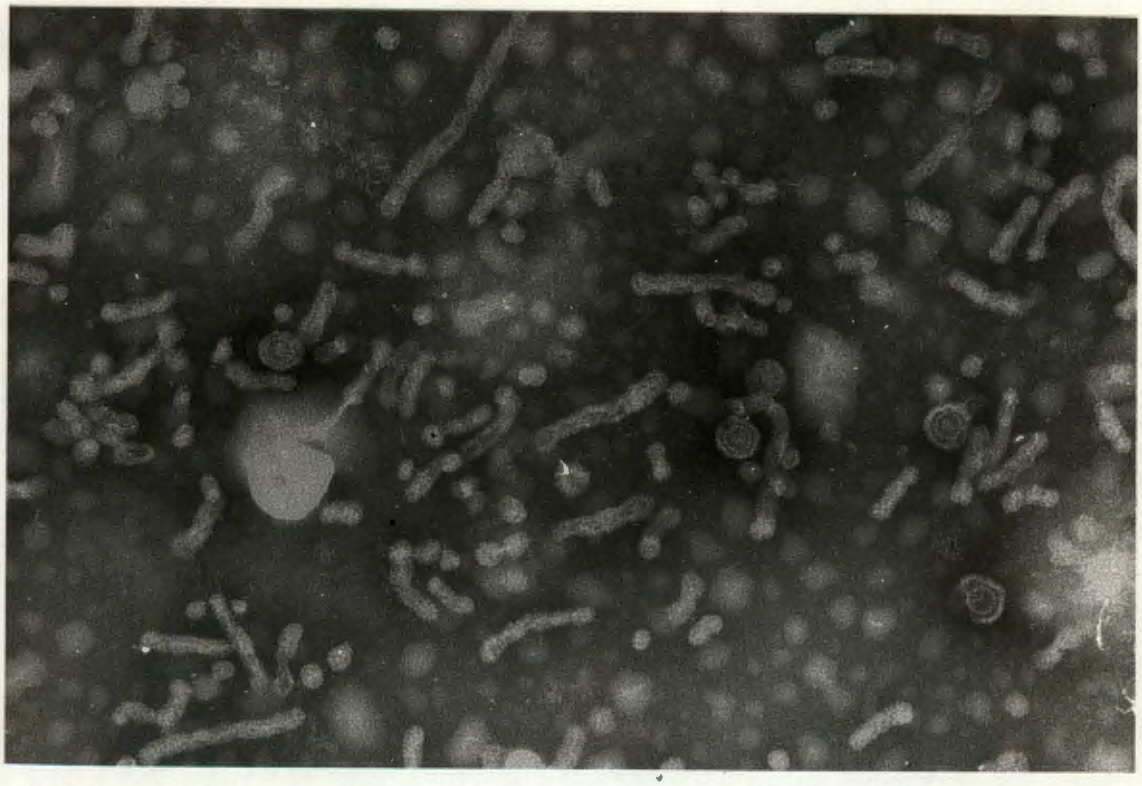
incubation with antiserum (Plate 7.2.a.) showed the characteristic "moth eaten" appearance of the particles. When anti-HBs was added to this preparation the filaments, spherical particles and Dane coats were similarly complexed (Plate 7.2.b.) and therefore aggregation was unaffected by the detergent treatment. Occasional free lying Dane core particles could be found with no evidence of attached gamma globulin.

Antibody to HB Core Antigen (anti-HBc)

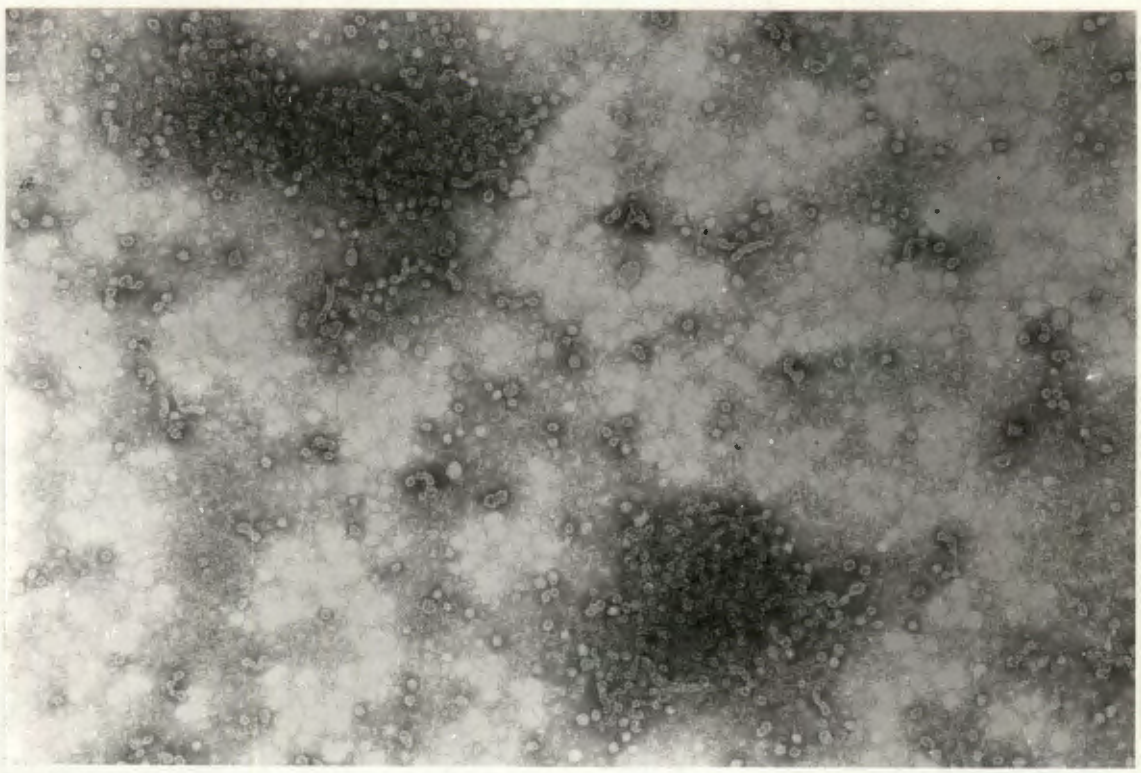
Antigen, pretreated with 1% Mucasol detergent, was incubated with an appropriate dilution of globulin containing anti-HBc obtained from a chronic carrier of HBsAg.

The electron micrographs (Plate 7.3.a. and b.) showed the presence of aggregates of Dane core particles, linked by immunoglobulin strands, with associated Dane coats but with unaggregated evenly distributed filaments and small spheres of surface antigen present in the preparation. The surface antigen showed the moth eaten appearance due to the effect of Mucasol treatment.

It is apparent that not all of the inner core particles have been completely released from the Dane coats which are present in varying degrees of disruption in the complexes. Where antigen was treated first with proteolytic enzyme followed by exposure to the detergent

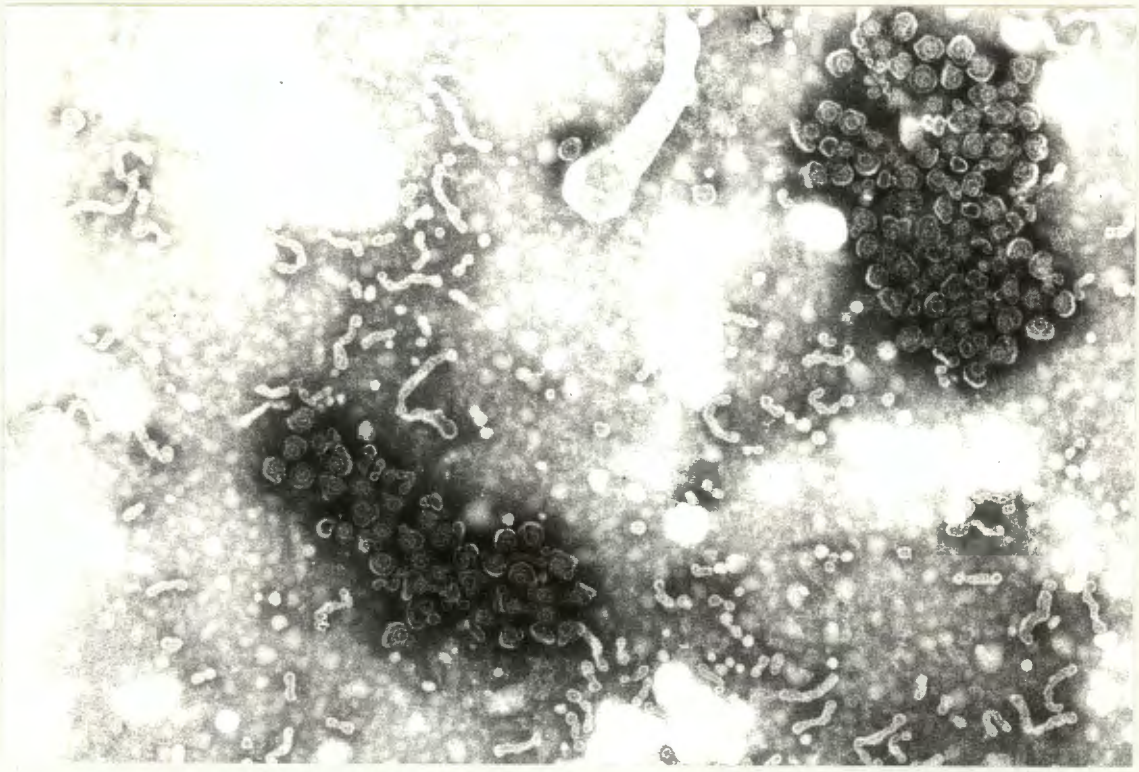


7.2.a. Mucasol control. Partially purified HBsAg treated with 1% Mucasol in PBS. Magnification x 120 000

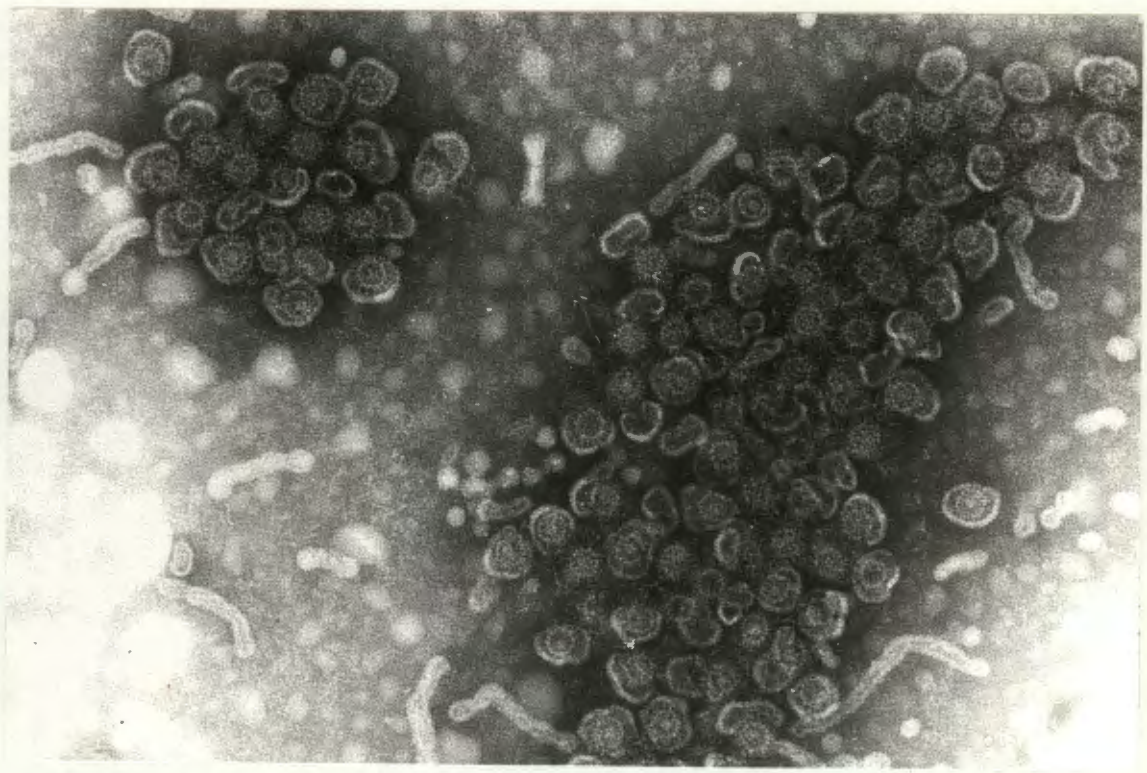


7.2.b. Mucasol treated HBsAg aggregated by baboon anti-HBs. Magnification x 80 000

PLATE 7.2.



7.3.a. Mucasol treated HBsAg incubated with globulin prepared from a chronic HBsAg carrier. Two HBcAg complexes are shown. Dane coats are seen within the complexes. Filamentous and small spherical HBsAg are unaggregated. Magnification x 80 000



7.3.b. A similar preparation to 7.3.a. Magnification x 200 000

PLATE 7.3.

and then subjected to anti-HBc, large complexes consisting entirely of core particles linked together by immunoglobulin were seen (Plates 7.4.a. and b.) without Dane particle coats.

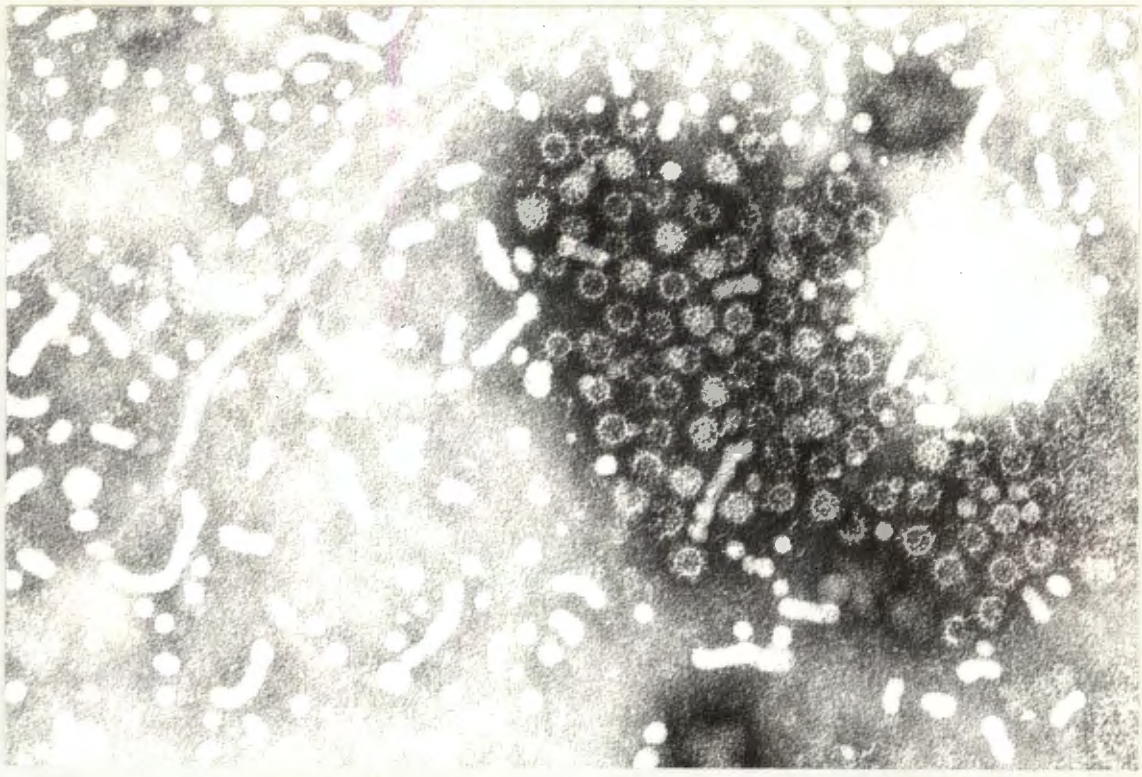
Exactly similar core complexes were seen when the same antibody was added to a suspension of Dane cores extracted from the nuclei of hepatic cells of a renal dialysis patient on immunosuppression.

Antibody to a Cryptic Antigen (anti-HB_{III})

Untreated serum from a chronic carrier frequently showed the presence of Dane particle complexes in which there were also 'tadpoles' consisting of Dane particles with attached tails of surface antigen and free tubular filaments as well (Plate 7.5.)

When the antigen was treated with a detergent, e.g. Mucasol or deoxycholate, and then mixed with serum from a chronic carrier, in this case the patient's own serum, damaged Dane particles (cores and coats) were found in large aggregates (Plates 7.3.a. and b.)

When antigen was treated first with protease 0.6 units/ml followed by detergent treatment and incubated with carrier globulin, large complexes of Dane cores occurred (Plates 7.4.a. and b.), but no Dane coats were present in the complexes.



7.4.a. and b. HBsAg treated with protease followed by Mucasol prior to incubation with globulin from the same serum. Two HBc complexes are shown (cf. Plate 7.3.). Dane coats are not involved in the immune complexes. Magnification x 120 000

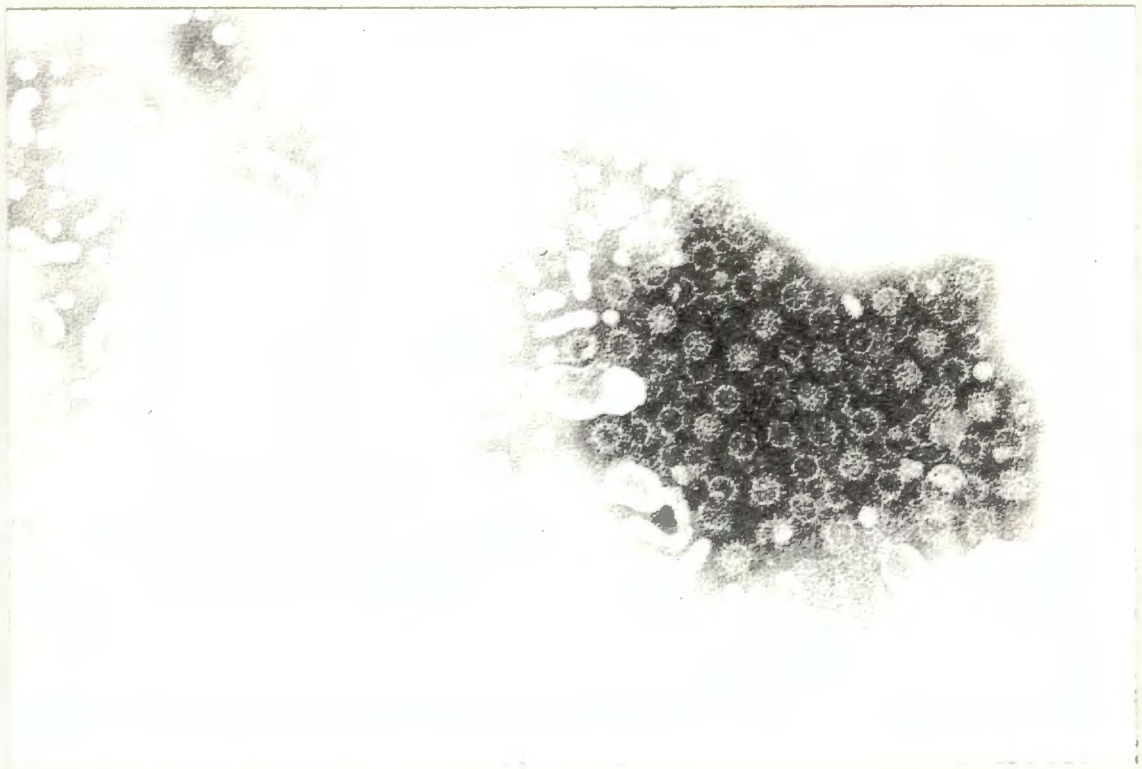


PLATE 7.4.

7.5. Untreated serum from HBAg positive blood donors. Dane particle complexes, including tadpole forms, demonstrate the natural occurrence of HB_{III}/anti-HB_{III} interaction. x 120 000

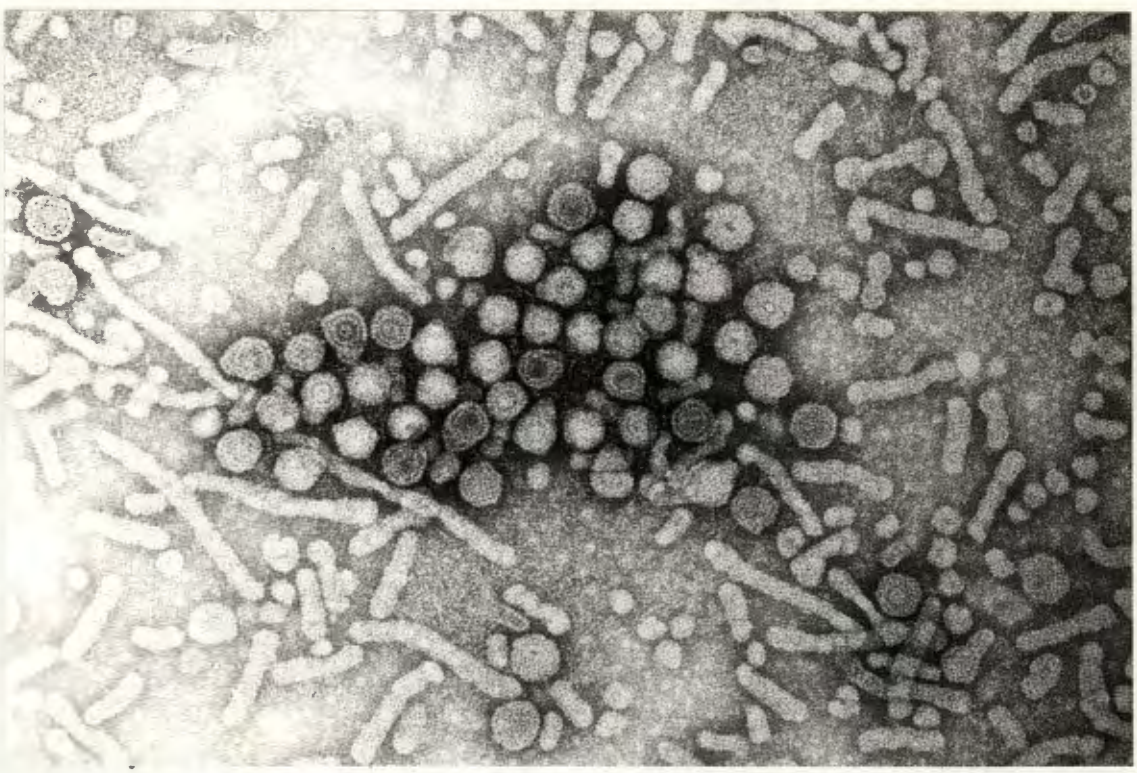


PLATE 7.5.

DISCUSSION

The demonstration by electron microscopy of an antigen-antibody reaction between the small spherical antigen, filaments and intact Dane particles (HBsAg) and specific antiserum (anti-HBs) is direct evidence of reaction which may be monitored by many serological techniques.

Hoofnagle et al., (1973) demonstrated antibody to the core of the Dane particle in all sera from chronic carriers. Serum from an immune blood donor, fractionated into IgG and IgM classes of antibody and incubated with a suitable core preparation demonstrated anti-HBc activity by immune electron microscopy (Stannard et al., 1973).

A preparation of Dane cores, prepared from liver cell nuclei or from circulating antigen by detergent release, was standardised to achieve near optimal proportions before deciding from the electron micrograph on the specificity of the reaction. Conditions of antibody excess produced small complexes, and occasional isolated cores, but in each case the cores were smothered by immunoglobulin strands. Antigen excess was somewhat misleading since core complexes were rarely present and the presence of anti-HBc on single cores could only be assessed by visually identifying attached immunoglobulin molecules. This required optimal resolution of the

specimen, a condition which was produced only with great technical difficulty and after painstaking preparative procedures. The zone of equivalence was shown by the formation of discrete complexes, the involvement of virtually all the specific antigenic structures in these complexes and the linkage of these structures by discrete antibody molecules. Once the appropriate dilution of antiserum has been determined in relation to the standardised quantity of antigen, the reaction was assessed for specificity. Minor modifications were introduced when determining the effect of detergents or enzymes but such variations of procedure were monitored by comparison with a control preparation adjusted for optimal proportions.

The presence of the third antigen-antibody system (anti-HB_{III}) was suggested by the occurrence in sera of circulating Dane complexes (Field and Cossart, 1971; Zalan et al., 1971; Stannard et al., 1973). It was further noted that treatment by detergent (1% Mucasol) or bile salt (0.25% deoxycholate) of antigen in the presence of antibody globulins from the sera of chronic carriers, produced very large aggregates of Dane particles. Closer examination of these aggregates showed that three Dane particle components, i.e. damaged but whole Dane particles, partially attached coats and separated coats, were all involved in this complex. In addition there were "tadpoles" consisting of Dane particles with attached tails of surface antigen

the globulins from the same serum were the result of at least two antibodies, anti-HBc and anti-HB_{III}. These formed combined aggregates with Dane particles linked by antibody to partially detached coats and to intact but unmasked coats. Partially released cores, still held by the coats, were in turn seen to be linked to detached cores and the whole mass interlocked into a single aggregate. (Plates 7.3.a. and b.). This would explain the occurrence of mixed aggregates in a system where separate specificities and antibodies should result in pure aggregates of each component. Further information in keeping with the hypothesis was obtained by examining the effect of a proteolytic enzyme, protease, on the mixed aggregates.

Protease digestion of partially purified antigen had no apparent effect on the morphology or antigenicity of the Dane coat. However, if protease was allowed to digest partially purified antigen under the same conditions, the aggregates produced after detergent treatment by incubation with homologous globulin did not contain Dane coats. Only detached cores were involved in the antibody reaction as shown by the presence of radially attached strands of globulin (Plates 7.4.a. and b.). Protease digestion must be considered to have altered or removed the determinant sites for anti-HB_{III} in the Dane coat wall, but the sites on the cores for anti-HBc determination remained unaltered. Neurath et al., (1976) detected determinant sites on damaged Dane

and "free tails" or tubular filaments of surface antigen but no small spheres (Plate 7.5.). It was thought that an antigenic site, located in the Dane coat material and the "tails", was revealed by the action of these agents. Damaged HBsAg of the small spherical form do not possess this cryptic antigen since these components remain unaggregated after detergent treatment and after exposure to the serum of chronic carriers.

The cryptic "HB_{III} antigen" of the Dane coat may be unmasked during the life of the Dane particle in the circulation, allowing reaction with anti-HB_{III}, and accounting for the presence of naturally occurring circulating small complexes of Dane particles. These were often seen in sera which contained Dane particles, but in each case the majority of the Danes were intact and unaggregated, the small numbers of aggregated particles are thought to represent a minority population nearing the end of their "life span". In the <u>in vitro</u> studies using partially purified (globulin-free) large component antigen, it was possible to treat the antigen with various agents and then return the patient's own immunoglobulin to assess his antibody response. It was found that lipid damage by brief chloroform extraction, anionic detergents (Tween 80 and Mucasol) and bile salts unmasked the cryptic antigen. The resultant damage also released the core from the coat which in turn initiated HbcAg/anti-HBc reaction. The complexes formed by detergent treated antigen followed by incubation with

particles, "tadpoles" and tubules using affinity chromatography and the "e" system. These authors suggested that these sites may be similar to those described (Moodie et al., 1974) in relation to anti-HB_{III}.

It may therefore be stated that the simple observation of an immunological reaction by electron microscopy resulted in the identification of reactive sites of the hepatitis B antigen without dependence on the isolation and purification of the antigenic sub-units. The occurrence of antibody in chronic carrier serum directed against a cryptic antigen present in Dane particles from the same patient may be of value in understanding and unravelling the complexities of this peculiar system of antigens and antibodies.

Chapter 8

SERUM PROTEINS AND HB ANTIGEN

INTRODUCTION

Purification of the hepatitis B antigen for analysis of protein and lipid components has been achieved by a variety of methods (Gerin et al., 1969; Millman et al., 1970; Dreesman et al., 1972, and many others) However these procedures have seldom been controlled by electron microscopy and have often utilised preparative methods that may well have caused unexpected, undetected and unintentional alteration of the surface of the antigen. These changes are particularly difficult to assess when some surface components appear to be normal constituents of human plasma. The extent of purification necessary to produce an HBsAg mono-specific antiserum (Boenisch and Katz, 1971), free from antibody cross reacting with normal serum components, may suggest that normal serum proteins are indeed present on the surface of the antigen in an association which may be part of its normal structure or consequent upon absorption. Certainly, HBsAg isolated from serum by a mono-specific antiserum in an affinity chromatograph column and inoculated into a sheep, produced an antiserum with antibodies to both HBsAg and to several normal serum components (Chapter 5). Two communications (Neurath et al., 1974; Burrell, 1975) reported the presence of human plasma proteins associated with HBsAg.

Neurath et al., (1974), using affinity chromatography, reported binding of HBsAg, as assayed by RIA, by antibodies to a variety of serum components and concluded that these antigenic determinants were an integral part of HBsAg. Burrell, (1975) using purified ^{125}I-labelled HBsAg, showed that HBAg particles have tightly bound antigenic determinants to a variety of serum proteins but was uncertain whether these were actually integrated into the structure of the HBAg particles. These observations were a little at variance with the early report of Millman et al., (1971) who, using the relatively insensitive technique of gel diffusion, failed to detect any reaction between purified antigen and antibody to serum components, but was able to demonstrate precipitin lines under the same conditions when the HBAg was treated with 1% Tween 80. This suggested that if the serum proteins were present as an integral part of the antigen, they were non-reactive unless first unmasked by the action of the detergent. However, this was not confirmed in the reports by Burrell, (1975) and Neurath et al., (1974) both of which claimed serum protein determinant sites on antigen not treated with detergent. Burrell did, however, report a loss of antigen after purification in 28% caesium chloride and for this reason used a gradient of sucrose in 14.3% caesium chloride. Neurath and his co-workers, on the other hand, prepared antigen by a procedure which included the use of a 10 - 50% glycerol gradient operative for 15.5 h.

Immune electron microscopy was chosen as the method for examining the effects of the serological reaction. Preliminary experiments showed that anti-human IgG could be used to identify IgG anti-HBs on the surface of hepatitis B antigen particles when used in a sandwich technique. The experiments, outlined below, were designed with a two-fold objective; to determine whether serum proteins were present on the surface of undamaged components of HBAg, and if not, whether treatment with detergent or glycerol or caesium chloride under the conditions employed by Burrell, (1975) and Neurath et al., (1974) could unmask any serum protein.

METHODS

HBAg was prepared for this series of experiments by sedimentation of a pellet from whole serum at 60 000 x g for 60 min resuspended in 0.06 M phosphate buffer, pH 7.2.

Experiment I

One "grid dose" of antigen in 0.25 ml of phosphate buffer was incubated with an equal volume of either (i) 1/20 dilution of goat anti-human gamma-specific IgG; or (ii) PBS, for 30 min at 37°C and thereafter prepared for electron microscopy by sedimentation and twice washing with phosphate buffer at 50 000 x g for 60 min.

Experiment II

"Grid doses" of antigen were suspended in

(i) PBS

or (ii) 1% Mucasol

or (iii) 15% caesium chloride

or (iv) 25% glycerol

and held at 4°C for 18 h. The antigen was sedimented at 60 000 x g for 60 min and resuspended in each case in 0.25 ml PBS and incubated with appropriate dilution (see Chapter 7) of anti-human IgG immunoglobulin. The antigen was washed by 2 cycles of centrifugation in phosphate buffer at 50 000 x g for 1 h before negative-staining with 1% phosphotungstic acid for examination in the electron microscope.

RESULTS

Experiment I

Hepatitis B antigen, sedimented from whole serum and incubated with anti-human IgG, was dispersed and uncomplexed and was identical in appearance with the control preparation in PBS shown in Chapter 7 (Plate 7.1.a.)

Experiment II

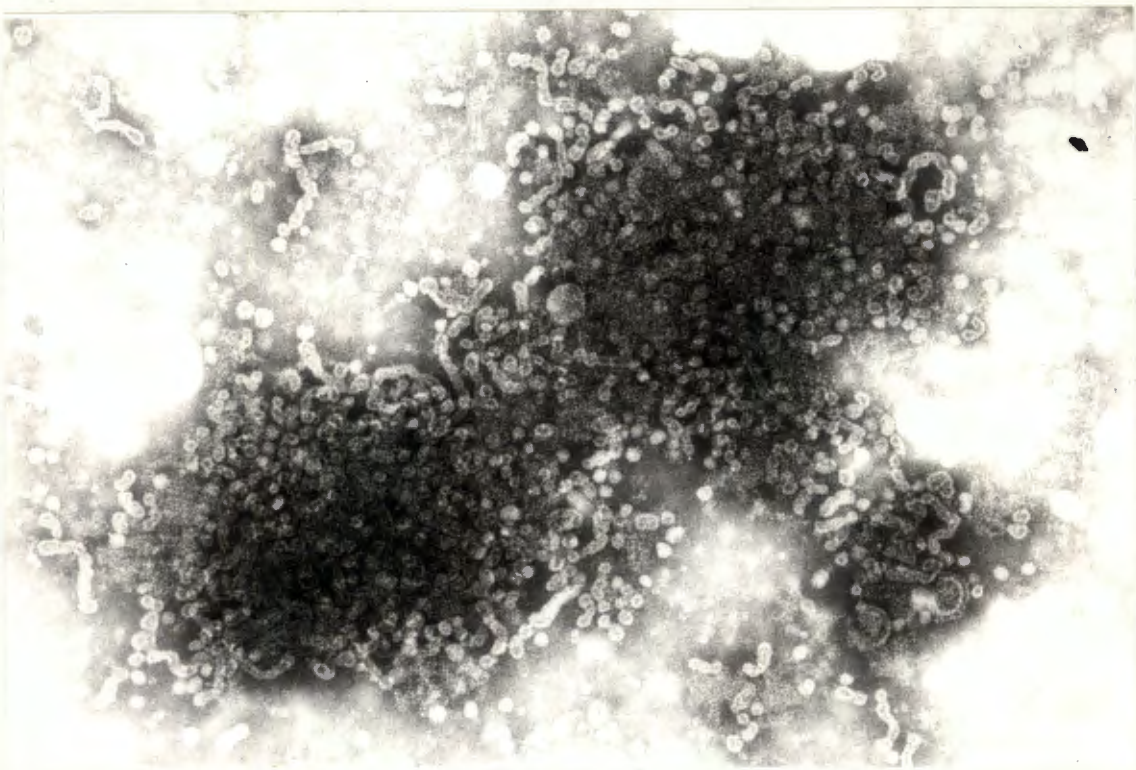
Detergent treated antigen showed the observed distortions of the HBAg components ascribed to this agent

(Chapter 7), but antigen treated with caesium chloride and glycerol showed no visible morphological changes. After incubation with anti-IgG, the untreated antigen (PBS incubated) again showed no evidence of complex formation. In sharp contrast with this, however, the detergent treated antigen was strikingly complexed by anti-IgG with involvement of spheres, filaments and Dane coats (Plate 8.1.a.). No free cores were seen in the complexes. HBAg particles which had been exposed to glycerol and caesium chloride were also obviously complexed by the anti-IgG (Plate 8.1.b.).

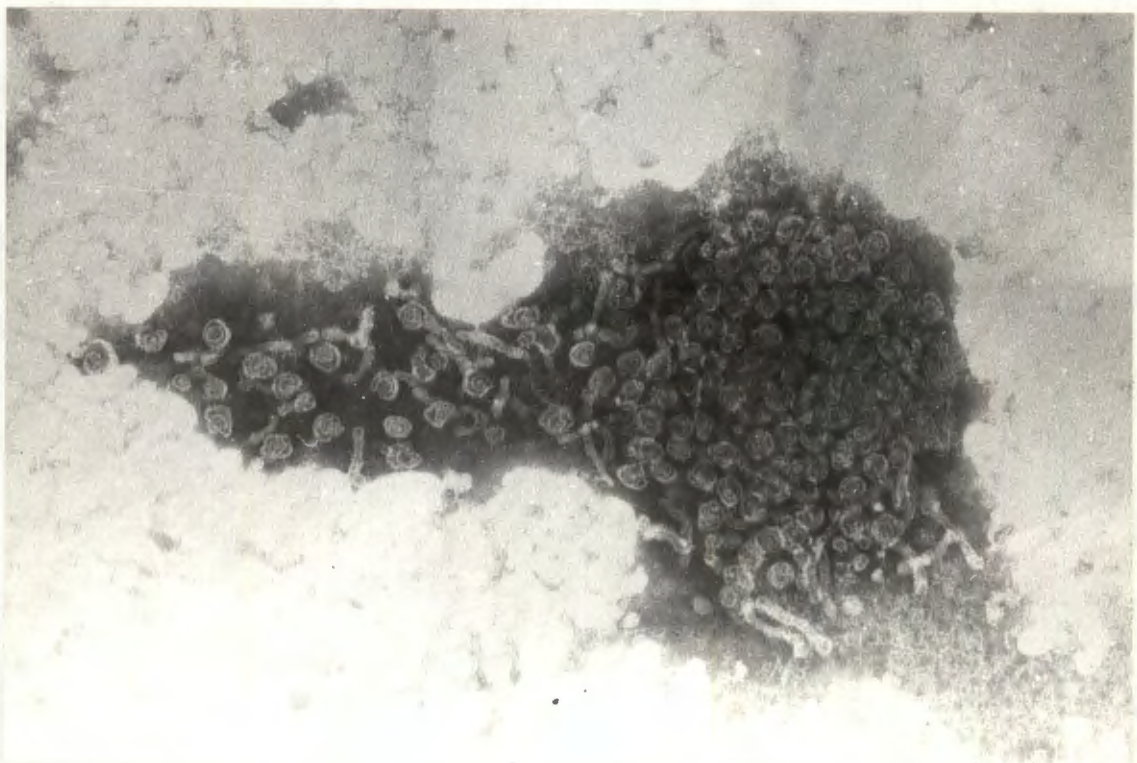
Elaboration of these experiments for further evidence of receptors on the HBsAg was undertaken with highly specific antisera, e.g. anti-albumen, anti-IgG (Fab), anti-IgG (Fc), anti-IgM (mu-chain specific) and anti-C_3. All these antisera complexed the treated antigen. All with the exception of anti-albumen and one source of anti-C_3, failed to complex undamaged antigen.

DISCUSSION

These results indicate that anti-IgG immunoglobulin does not form complexes with undamaged antigen. Antigen prepared by simple centrifugation cannot be considered to be absolutely pure. If IgG is present as a surface contaminant of the partially purified antigen, this was not detected by the methods used in these



8.1.a. Mucasol treated antigen incubated with anti-human IgG. A large immune complex consisting of Dane coats, filamentous antigen and small spheres. Magnification x 80 000



8.1.b. Caesium chloride treated antigen incubated with anti-human IgG. A large immune complex consisting almost entirely of Dane particles. Magnification x 80 000

PLATE 8.1.

experiments.

On the contrary, the same antigen, after mild disruptive treatment, was firmly complexed by anti-IgG. Using anti-IgG as a marker, HBAg prepared from stored whole serum by centrifugation, has a receptor site for anti-human IgG, a site which is only unmasked by disruptive treatments. This receptor site is an integral part of the structure of the HBsAg and however masked, is sufficiently integrated with the antigen itself to resist dislocation or loss by moderately harsh treatment. The work of Burrell, (1975) and Neurath et al., (1974) remains valid concerning the affinity of various antisera to human proteins for HBAg, but the present experiments now appear to dismiss any suggestion that these serum components are not integral to the structure of the antigen. The technique of immune microscopy enables the reaction between receptor site and antiserum to be made at a morphological level.

The detection of cross-reacting sites to these human proteins corresponds well with the proteins detected on HBAg by Millman, Neurath and Burrell, (1975). Albumin was found in the experiments in this study to be present on the surface of the untreated antigen and persisted after detergent treatment.

The antigen used in the tests was stored at 4°C for a period varying between 2 months and 4 years and in this

respect, therefore, differs from naturally circulating antigen. It is possible that the conditions of storage were responsible for changes in the antigen to render sites inaccessible to antisera, and that this masking is removed by the methods described. However, evidence for the presence of immunoglobulin components and activated complement determinants "within" the morphological structure of the HBsAg is convincing and may have an important significance.

Chapter 9

ENZYME AND DETERGENT EFFECT ON HB ANTIGEN

INTRODUCTION

Until a decade ago the distinction between serum hepatitis and infectious hepatitis was made by consideration of the route of infection. This inferred that hepatitis B was transmitted entirely parenterally. Evidence that non-parenteral spread occurred in closed populations and between intimate contacts is presented in Chapter 1.2. The significance of these findings highlighted the search for antigen in the faeces and in all secretions such as tears, urine, bile, intestinal secretions, menstrual fluid and saliva.

Using standard serological tests for the detection of HB antigen Grob and Jemelka, (1971) reported positive results in faecal extracts from all of eleven patients with acute hepatitis. Cossart and Vahrman, (1970) were unable to find HB antigen in faecal extracts from patients with acute hepatitis after 100-fold concentration of the extracts, and Gust et al., (1970) reported 47 negative results from 15 patients with antigenaemia. More recently Irwin et al., (1975) were unable to confirm the presence of HBAg in the faeces of 11 patients with acute hepatitis B or in any of 8 carriers.

At the upper end of the alimentary canal two reports (Serpeau et al., 1971; Akdamar et al., 1971) indicated that hepatitis B antigen could be detected, again by serological methods, in bile but not in gastric or duodenal secretions. This, although no such inference was drawn by the authors, seemed to suggest that HBAg present in bile, was no longer detectable when the bile-containing-antigen was excreted into the duodenal juice.

It is possible that dilution of the bile lowered the concentration of antigen to below the threshold of detection but other possibilities exist. The reports of Piazza et al., (1973) and Grabow and Prozesky, (1973) indicate the presence of an inhibitory factor in the gastro-intestinal tract. Both of these groups independently found that if HBAg was incubated with faecal extracts at 37°C, the previously detectable antigen no longer gave positive results in their indicator systems. The inhibition did not occur if the temperature of the incubation was 2 - 4°C. In addition Piazza et al., (1973) found that human small bowel mucosa similarly inhibited the demonstration of hepatitis B antigen. Gastric juice had no effect on the detection of antigen but acholic faeces had the same effect as normal faeces.

From these reports it appeared that an enzyme (or enzymes) could be responsible for the disappearance of serologically reactive antigen, particularly as the reaction was stated to be temperature dependent.

Hirschman et al., (1973) described the morphological effect of enzyme on preparations of HBAg but other workers found the antigen to be resistant to digestion by a wide variety of enzymes (Rao and Vyas, 1974).

This conflicting evidence, together with an awareness of the two major antigens of the Dane particle, prompted the performance of a series of experiments designed to test the opposing concepts by the construction of an in vitro model of the gastro-intestinal tract.

METHODS

In each of the following experiments the antigen used was produced as follows: Sucrose 5% was added to 2.0 ml of the 10% re-precipitate fraction produced by PEG displacement. (Fig. 2.1.c.). This was chromatographed on a Sepharose 4B column (20 x 2.5 cm) at a flow rate of 12.5 ml per h. The eluate was monitored by u-v absorption at 280 nm and the initial 5.0 ml corresponding to the first peak after the void volume was collected (Fig.3.1.c.). This fraction was centrifuged at 60 000 x g for 90 min and the antigen was resuspended in the enzyme or reagent as stated in the various experiments.

Experiment 1

(i) The antigen was resuspended in 2.0 ml bile. The bile was obtained from a post-cholecystectomy T-tube

and was shown to be free of hepatitis B antigen by electron microscopy.

The mixture was kept at 4°C overnight.

(ii) The antigen was resuspended in Na deoxycholate 0.5% w/v H_2O ($C_{23}H_{39}O_2$.COONa Hopkins and Williams, Chadwell Heath, Essex, England) and kept at 4°C overnight.

(iii) The antigen was resuspended in protease 0.6 units/ml H_2O (Streptomyces griseus, Sigma) and incubated at 37°C for one h.

(iv) The antigen was resuspended in 2.0 ml of a faecal extract, divided into 2 samples of which one was incubated at 37°C for 1 h and the other at 37°C for 18 h. The faecal extract was prepared by emulsifying 10 g of fresh faecal material in 10 ml PBS. This suspension was clarified by cycles of low speed centrifugation and a final cycle of 50 000 x g for 60 min produced a clear yellow supernatant fluid for use as a faecal extract.

In each case after the relevant treatment, the mixture was centrifuged at 50 000 x g for 60 min and the pellet washed by centrifugation twice before examination by electron microscopy.

Experiment 2

Deoxycholate-treated-antigen, obtained from the procedure described in Experiment 1 (ii), was digested with protease under the following conditions:

(i) The deoxycholate-treated-antigen was washed by centrifugation at 50 000 x g for 60 min, suspended in

2.0 ml of distilled water containing 12 units of protease and incubated at 37°C for one h. A control preparation in which deoxycholate-treated-antigen was incubated in 2.0 ml distilled water without protease, was included.

(ii) To unwashed deoxycholate-treated-antigen, 12 units of protease was added and the mixture was incubated at 4°C for 1 h. A control specimen without protease was included in the experiment.

(iii) Preparations identical with those in (ii) above were incubated at 37°C for one h.

To samples in which core particles were sought, a further step was included. The centrifuged antigen was resuspended in 0.25 ml PBS to which 0.25 ml of anti-HBc globulin was added. This was incubated for 1 h at 37°C and for 19 h at 4°C. The final preparation was prepared for electron microscopy by washing with three cycles of centrifugation.

Experiment 3

Deoxycholate-and-protease-treated-antigen was produced by the method of Experiment 2 (iii) above. Faecal-extract-treated-antigen was produced by the method of Experiment 1 (iv). A control specimen in which PBS was used instead of the deoxycholate and protease was included.

These two samples were centrifuged, washed and finally suspended in 0.3 ml PBS. One dimensional rocket

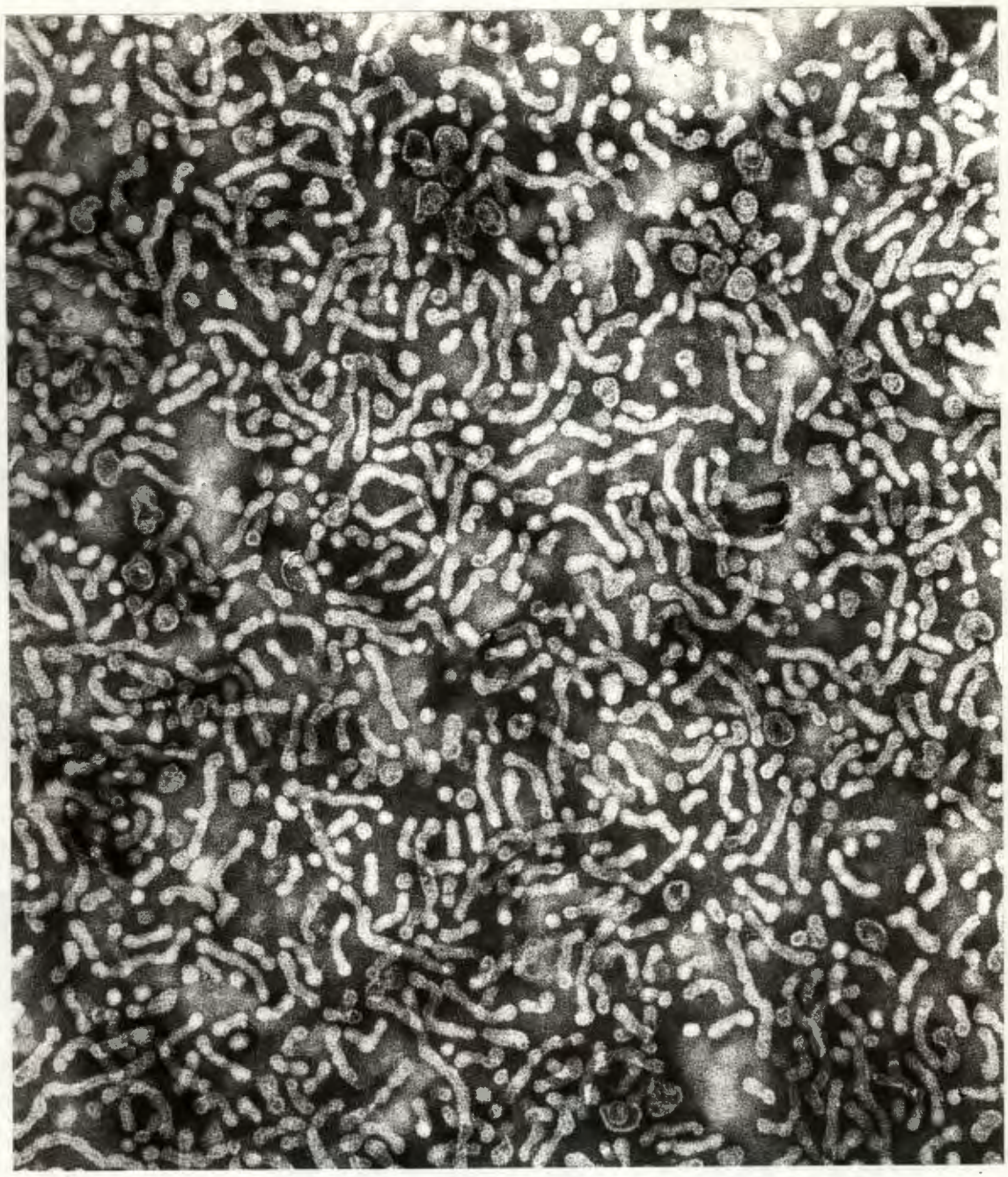
Laurell immuno-electrophoresis was performed in a 1% agarose gel in which baboon anti-HBs was incorporated.

RESULTS

Experiment 1

Partial purification of the antigen by PEG precipitation and Sepharose chromatography produced a fraction in which the antigen was removed from the majority of the serum components and in particular from the IgG anti-HBc. This combination of methods resulted in a supply of antigen, which although not pure, was considered adequate for determining the effect of bile or bile salts and enzymes on both the surface antigen and the inner core and the outer coat of the Dane particle. (Chapter 4).

On electron microscopy both bile and deoxycholate-treated-antigen showed a similar "detergent effect". (Plate 9.1.). The small spherical antigen had rarefied centres, the filamentous forms were segmented and no intact Dane particles were seen. The Dane particles were seen in various stages of uncoating due to the "detergent effect". Characteristically those most easily found on the screen occurred in small collections in close proximity to one another as a result of the "detergent effect" on circulating Dane complexes. There were no antibody molecules linking the Dane cores. This confirmed



Globulin free HBsAg after Na deoxychlate treatment. Small spheres are rarefied and penetrated by stain. Some filaments are seen to be segmented and appear to break into small spheres. Most Dane particles are penetrated by stain, some have released central cores. Small groups of Dane particles indicate the effect of detergent on circulating Dane complexes. No antibody molecules are attached to released Dane cores. Magnification x 100 000

PLATE 9.1.

the removal of the IgG anti-HBc and the absence, in this carrier serum, of anti-HBc IgM activity.

Digestion by protease at 37°C for 1 h had no visible effect on the antigen as assessed by the unaltered morphology on electron microscopy.

Faecal-extract-treated-antigen showed "detergent effect" and free cores could be demonstrated by the addition of anti-HBc. The antigen after incubation at 37°C for 18 h showed considerably more damage and few filamentous forms were seen. An occasional damaged Dane particle could be detected.

Experiment 2

The deoxycholate-treated-antigen which had been washed free of the bile salts when incubated at 37°C for 1 h with protease did not differ in morphology from the control to which no protease had been added. Both samples showed the "detergent effect" of the bile salt as described in Experiment 1 above, but no additional changes were seen in the sample which had been digested with protease. This was in sharp contrast to the action of protease in the presence of deoxycholate. Mixtures incubated at 37°C for 1 h showed that no intact surface antigen remained; the spheres, filaments and Dane particle coats had largely disappeared or were morphologically unrecognizable as antigen. An occasional Dane core

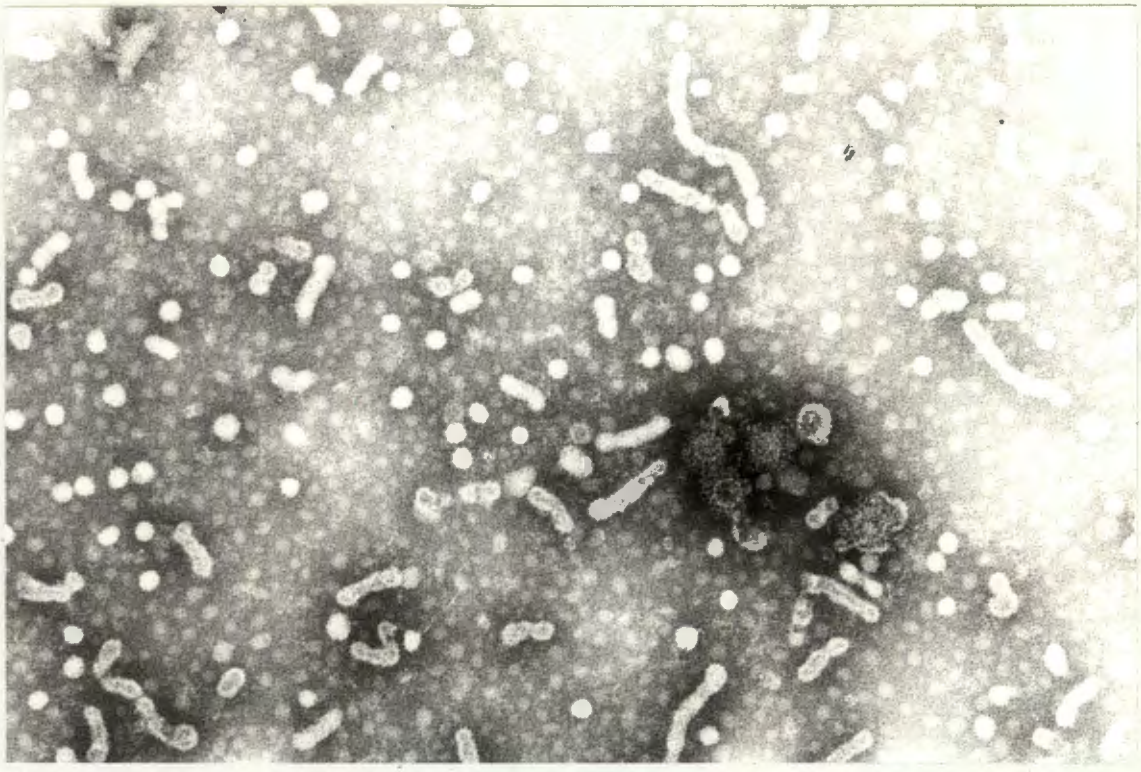
was found among the debris. The addition of core antibody to such a sample resulted in the formation of complexes of these scattered Dane cores, and small aggregates of 7 - 11 cores linked by visible antibody molecules were more easily seen. (Plate 9.2.b.). A few intact small spheres were present after incubation with the anti-HBc, but these spheres did not show any "detergent effect", thus indicating that this antigen represented HBsAg present in the anti-HBc prepared from carrier serum.

Experiment 3

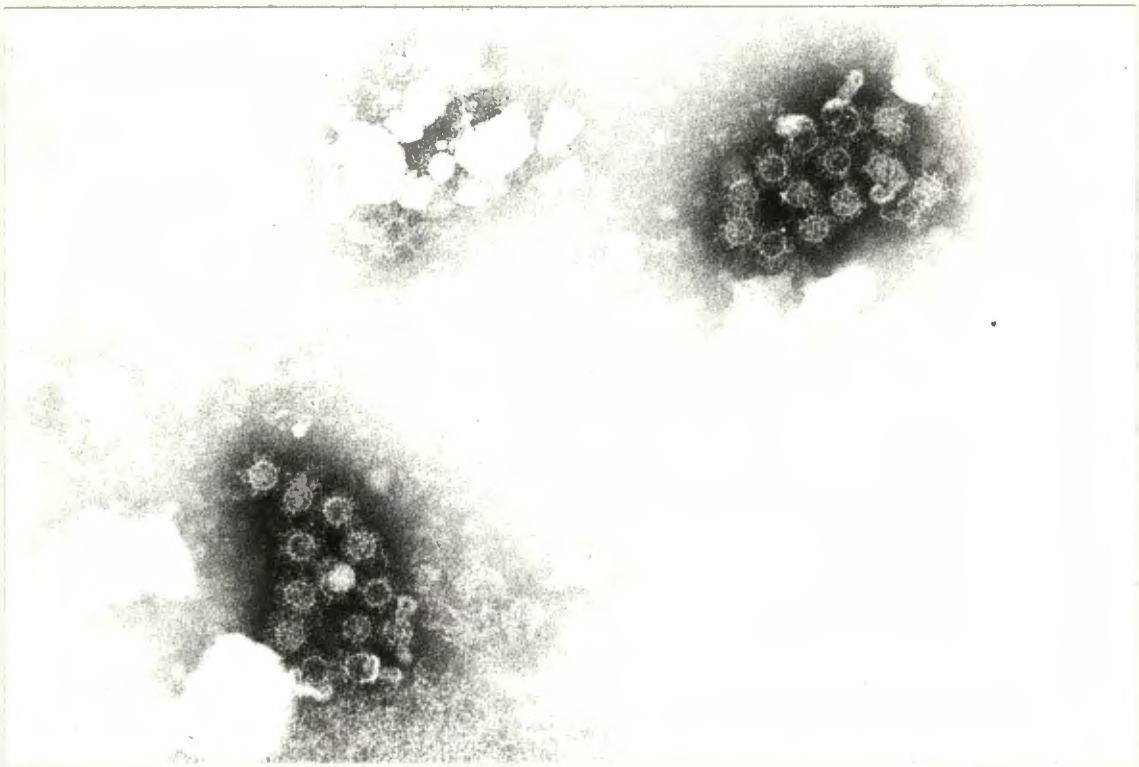
No precipitin line was formed in the gel by the deoxycholate-and-protease-treated-antigen. The faecal-extract-antigen and the control specimen produced the usual rocked shaped precipitate in one dimensional Laurell immuno-electrophoresis.

DISCUSSION

Bile and sodium deoxycholate have a similar effect on the antigen. Once this was established the single bile salt was used in subsequent experiments. Many trial digestions were performed and examined. Those outlined above were representative of key points in the development of the "in vitro" model of the intestinal tract.



9.2.a. Detergent treated HBsAg-containing serum. Released Dane cores with radially attached antibody. HBsAg shows mild detergent effect. Magnification x 120 000



9.2.b. Partially purified HBsAg following protease digestion in the presence of deoxycholate. Anti-HBc added to form immune complexes from scattered free cores. HBsAg no longer present (cf.9.2.a.). Magnification x 120 000

PLATE 9.2.

It may be assumed that antigen present in the gall bladder and hepatic ducts, whether actively secreted with the bile or released into the bile space as a result of cell lysis, will be subject to detergent effect. This effect was absolutely predictable whether bile, sodium deoxycholate or Mucasol was used and has been reported with other detergents such as Nonidet P 40 and Tween 80 (Almeida et al., 1971).

The detergent-treated-antigen was found to react with anti-HBs both in gel-precipitin systems and on immune electron microscopy. Reports of the detection of antigen in bile or duodenal juice immediately after induced contraction of the gall bladder are easily understood since under these conditions antigen is exposed to the detergent effect of the bile alone.

Protease (not a single enzyme) was used as a representative of the bacterial enzymes present in the bowel. It was felt that these, rather than the secretory digestive enzymes, were more likely to be the enzymes present in active form in faecal material and it was this that was twice reported as having an inhibitory effect on the detection of antigen (Piazza et al., 1973; Grabow and Prozesky, 1973).

Protease alone had no visible effect on the antigen, nor did digestion by protease of detergent damaged antigen after washing away the detergent; but

protease in the presence of the bile salt virtually destroyed all of the surface antigen. No reactive soluble antigen was demonstrated in an adequately controlled one dimensional Laurell electrophoresis.

Simple faecal extracts of various origins were applied to the HB antigen and in every instance the antigen showed the detergent effect. Enzyme activity in the small and large intestine occurs in the presence of bile salts (detergent) yet there was no apparent loss of surface antigen by quantitative Laurell electrophoresis. This is at variance with published reports (Piazza et al., 1973; Grabow and Prozesky, 1973) of faecal inhibitors, but the demonstration of the efficiency of a single bile salt and bacterial enzyme in destroying the surface antigen was felt to indicate adequately that surface antigen could not survive passage from the duodenum through the intestinal tract. It is possible that antigen may leak into the lumen with small haemorrhages in the lower bowel, particularly if raised portal pressure due to liver damage is present. The relatively brief exposure of this antigen to the intestinal contents would allow the antigen to remain reactive to serological identification.

The Dane core was found to survive under these experimental conditions and to retain its antigenic identity in immune electron microscopy. Recently Grabow et al., (1975) stated that they were no longer able to detect these cores after prolonged digestion with their

enzyme preparation.

Hepatitis B surface antigen thus does not survive enzymatic digestion in the presence of a detergent and except for added blood components, it is presumed that hepatitis B surface antigen would not be detected in faecal samples.

CONCLUSIONS

From the start it was appreciated that a ready supply of hepatitis B antigen was an inescapable prerequisite. Human plasma was the obvious starting material but since the Dane particle (Dane et al., 1970) had acquired a notoriety as a possible candidate for the title of hepatitis B virus, it was essential also to have a convenient supply of Dane particles. This was the first and the most difficult problem.

Dane particles could be demonstrated in 30% of plasma from local chronic carriers but in most instances they represented only a small proportion of the total hepatitis B antigen. So it was necessary to find a way of separating and concentrating the various components of HBAg.

Of particular interest was the important source of Dane core particles from the liver cell nuclei of a patient dying of acute fulminating hepatitis B. Such material was made available for diagnostic purposes in the early days of the investigation. The sample consisted of 3 - 4 g of liver tissue, which was homogenized and shown to have a high concentration of Dane core particles with practically no detectable surface antigen. Regretfully the rest of this post mortem liver was incinerated before it could be possessed.

It was presumed that another post mortem liver source of Dane core particles would soon appear, but unfortunately no further material of this nature appeared during the 4 years of this research study. This meant in effect that Dane core particles would have to be fractionated and concentrated from the plasma of chronic carriers.

Tissue sources other than the liver proved to be unrewarding.

All efforts were thereafter confined to plasma units from chronic carriers kindly sent to the laboratory from the Western Province Blood Transfusion Service. Some donors contributed more than one donation for this research programme.

The use of polyethylene glycol for concentrating and selectively precipitating hepatitis B antigen from serum became a routine procedure when quantities of HBAg larger than grid doses were required. Antigen produced by displacement and antigen sedimented directly from plasma by ultracentrifugation were morphologically and serologically indistinguishable. This was in contrast with the behaviour and appearance of HBAg after exposure to low pH, detergent or caesium chloride. Apart from the convenient concentration in one fraction of those components of plasma that were under investigation (all the large component antigen and all the gamma globulins),

precipitation with polyethylene glycol was occasionally the only method by which HBAg could be recovered from dilute solutions without concentration of salts and small molecular weight proteins. The failure to isolate HBAg completely from serum proteins or to achieve a greater degree of morphological separation of the components of HBAg by this independently developed technique, must be judged by comparing it with the results of the techniques used by Johnson and Newman (1972); Neurath et al., (1973)a and Nath et al., (1976). These workers, using differing conditions of pH and ionic strength, confirmed the preliminary nature of this preparative procedure. De Rizzo et al., (1972) following the method of Polson et al., (1964) used 8% PEG 6 000 at pH 4.6 to precipitate HBAg together with the alpha-globulins, (fibrinogen) and a minor proportion of albumin and gamma globulins. This step was followed by 3 cycles of absorption, washing and elution of the HBAg onto and from polyelectrolyte 60 to give a preparation suitable for application to more sophisticated purification techniques. The HBAg produced by this efficient large scale method retained serological activity, but no morphological studies were reported. Evidence presented in this thesis would suggest that considerable damage to large components of the antigen would result from the low pH PEG precipitation.

The use of PEG in this work at a safe pH with diluted serum, and the adoption of a reprecipitation step, was found to give exactly reproducible results when

assessed by the distribution of serum proteins (Plate 2.1.a.)

The second requirement for preparing Dane particle antigen called for removal of coexisting antibody from the preparation. The considerations which were given priority when choosing techniques, were the avoidance of any harsh process detrimental to the antigen and the ability to use the method for large scale processing. These were felt to be essential if sufficient antigenic material was to be prepared for serological techniques in use at the time. The basic methods of sedimenting and chromatographing concentrated fractions were usefully employed for immune electron microscopy; the development of batch ion-exchange method satisfactorily separated large amounts of antigen from globulin. When the final antigen fraction (Plate 3.2.a.7.) was treated with Mucasol, not only were antibody free Dane cores produced but electrophoresis in agarose gel demonstrated total absence of the residual β-lipoprotein component.

There are many methods of isolating relatively small amounts of HBAg, but the large scale ion-exchange batch method developed in this study proved to be neither totally destructive of the large components (low pH) nor apparently damaging to the surface antigen (caesium chloride, glycerol).

The relative inability to attain a clear cut

separation between Dane particles and filamentous antigen, and small spherical particles, by PEG displacement, even when small increments were used, indicates that a factor additional to Stokes radius was operative. Similarly sized particles with identical surface charge densities should be excluded by a single concentration of the polymer. The presence of small spheres in the fractions precipitated by a low concentration of PEG indicated that these particles must differ from their soluble companions. The difference was shown not to be one of size. Reports on the heterogeneity of purified HBAg in electrophoretic migration (Kim and Tilles, 1973) and the extensive analysis of the charge differences by Howard and Zuckerman, (1973) of small spheres of significantly similar diameter confirmed that this was indeed the reason. This was proved by Howard and Zuckerman, (1973) by electrofocussing HBAg in a gradient of carrier ampholytes a process which resulted in the isolation of pure HBAg from a partially purified (gel filtration) fraction.

Zone electrophoresis of serum in the apparatus of Polson and Russell, (1967) confirmed the alpha$_2$-beta globulin migration of HBAg, and indicated that separation of the antigen from serum proteins by gradient electrophoresis could only be achieved by electrofocussing with the aid of an ampholyte gradient. The detection of immune complexes in the most slowly migrating HBAg positive fractions has also been recorded with Allerton virus (Polson and Kipps, 1966).

The specially designed preparative immunoabsorbent technique (H-tube) was used initially to isolate HBAg from a partially purified PEG fraction (Moodie and Polson, 1973). It was subsequently found that whole serum could be applied to the column to achieve a one-step isolation procedure. Recent work on plant viruses indicates that the effect of hysteresis can be abolished by isolating the reaction gel from the wall of tube by suspension in a hydrophobic mixture of organic solvents. The important potential of the system for isolating abnormal components from normal human fluids or secretions has not yet been fully assessed.

The ability of sheep to react strongly to a large antigenic molecule such as lipoprotein and IgM (Harboe and Inglid, 1973) determined the choice of this species for raising an antiserum to HBsAg. The purification procedure for obtaining HBsAg was deliberately tailored to avoid alteration of its morphological and immunological properties. Antigen purified by affinity chromatography with baboon anti-HBs and free from contaminant human serum proteins when assessed by immuno-electrophoresis, nevertheless produced antibody against several human proteins when used to immunize a sheep. Rather than indicting the procedure for this "impurity", it would appear that active disruptive treatment is necessary for the detachment of closely associated serum proteins from the surface of serologically reacting HBsAg. Many reports of inoculation of "purified" HBAg into rabbits,

(Melartin and Blumberg, 1966) mice, (Millman et al., 1970a) guinea pigs, (Purcell et al., 1970) horses, (Cabasso et al., 1971) and sheep, (Duimel et al., 1972) relate that the majority of antisera produced required absorption with normal human serum proteins (NHS). Indeed one ingenious method was developed to overcome this difficulty by inducing tolerance to NHS before immunization with partially purified HBsAg (Madalinski et al., 1971). Dreesman et al., (1972) investigated three preparative methods, including low pH dissociation and ultrasonication, for isolating pure HBsAg. Each method produced an HBsAg which, when inoculated into goats, raised an antiserum with anti-NHS activity. Some of these antisera were acceptable for use as a monospecific reagent after dilution, but others required absorption with NHS. Maupas et al., (1976) found that the eluate from an anti-HBs-cyanogen-bromide-activated Sepharose column contained residual NHS components. These were removed by passage of the eluate over an immunoabsorbent coated with anti-NHS antibodies. This product was treated with formalin and used as a vaccine in chimpanzees and humans.

Normal human serum components in trace amounts in a vaccine for primate use are probably not important and affinity chromatography yields a preparation of adequate purity for this purpose.

Study of the morphological and serological effect of bile and faecal extract on HBAg, and the use of

deoxycholate and a bacterial enzyme (protease) as representatives of some of the digestive processes encountered in the intestinal tract, was prompted by conflicting reports of detection of HBAg in faeces. These experiments suggested that HBsAg was totally destroyed by these agents (Moodie et al., 1974a) and that the possible infectivity of faeces would be judged by detection of Dane core particles. No such particles could be demonstrated in faecal samples and Grabow et al., (1975) demonstrated the disappearance of Dane core particles after exposure to a different set of bacterial enzymes (carboxypeptidase).

In spite of the increasing frequency of electron microscopic examination of faeces for hepatitis A antigen and for infectious viral diarrhoea agents (rotaviruses) there have been no further reports, (as far as is known) of faecal HBAg.

Immune electron microscopy was extensively employed in assessing the antigenic specificity of the cryptic antigen of the Dane coat and in detecting anti-human serum receptors on the surface antigen. The technique of immune electron microscopy is well established and reports in other fields (such as hepatitis A) indicate the acceptance of this method. The demonstration of naturally occurring Dane complexes (Stannard et al., 1973) and the monitoring of the development of methods for the artificial reproduction of this reaction were performed

by electron microscopy. Artificial exposure of the cryptic antigenic site on the Dane coat resulted in a reaction with an antibody present in the serum of a chronic carrier (Moodie et al., 1974). This system is felt to be operative in all hepatitis B infections as an expression of the host response to degraded Dane and "tadpole" particles. Neurath et al., (1976) have convincingly demonstrated by immune electron microscopy that antibody to e-antigen, when incubated with Dane particle containing HBAg, complexes Dane coats and filamentous forms of the antigen. This is at variance with the suggestion of Nielsen et al., (1974) that e-antigen is related to the core component of the Dane particle. It is also startlingly different from the characterization of e-antigen as a small soluble protein (Magnius, 1975). The recent brief communication (Neurath and Strick, 1977) indicating that e-antigen may be an antibody will reconcile these two views.

The implications of this recent letter have not been assessed in this work, but investigation of the cryptic antigen (HB_{III}) as a receptor site for e-antigen would clarfiy the suggestion of Neurath et al., (1976) that the HB_{III} and e-systems are related.

Of equal clinical significance is the demonstration of IgG components within the surface of the HBsAg (Stannard and Moodie, 1976). This investigation has been extended to show that both Fab and Fc fractions of IgG,

IgM and C_3 are exposed by detergent treatment of HBAg prepared from stored serum. An earlier paper by Madalinski et al., (1974) described the absorption of partially purified heat treated (80°C for 1 h) HBAg to DEAE-cellulose at high pH. IgG, IgA and IgM were eluted from this preparation by a salt and pH gradient. These eluted antibodies were shown to be specific for HBsAg by precipitin reactions in gels. No C_3 was detected in the eluate.

The electron microscopic findings described in this work confirm that after some minor degree of damage, immunoglobulin determinants may be identified in close association with the HBAg; in addition C_3 was identified in this situation. It is felt that this gives some support to an hypothesis of continuous production of anti-HBs by the host under conditions of antigen excess. This reduced humoral response results in a stable association between these components and HBsAg. An association which in active hepatitis and in the chronic carrier, is unable to saturate HBsAg determinant sites or to cross link the majority of particles. A shift in balance towards recovery is heralded by circulating immune complexes and finally by clearance of the complexes from the circulation and elimination of infected hepatocytes.

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